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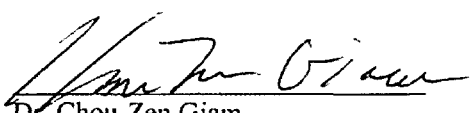


DISSERTATION APPROVAL FOR THE DOCTORAL DISSERTATION IN THE EMERGING
INFECTIOUS DISEASES GRADUATE PROGRAM

Title of Dissertation: "Functional Expression, Ephrin Receptor Tropism and Heterotypic Functionality of the Attachment and Fusion Glycoproteins of Cedar Virus, a Newly Discovered Henipavirus"

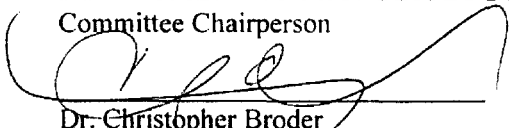
Name of Candidate: Stephanie Petzing
Doctor of Philosophy Degree
August 20, 2012

DISSERTATION AND ABSTRACT APPROVED:

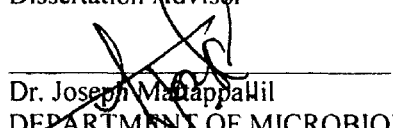

Dr. Chou-Zen Giam
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
Committee Chairperson

DATE:

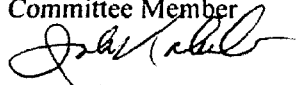
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Dr. Christopher Broder
DEPARTMENT OF EMERGING INFECTIOUS DISEASES
Dissertation Advisor


8/20/12


Dr. Joseph M. Appalil
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
Committee Member

8/20/12


Dr. Johnan Kaleeba
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
Committee Member

8/20/12


Dr. Joel Lowy
PROFESSOR, AFFRI
Committee Member

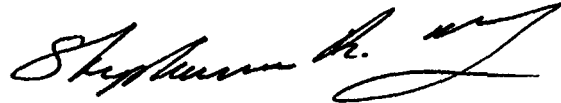
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Stephanie R. Petzing
Emerging Infectious Diseases,
Microbiology & Immunology
Uniformed Services University
09/25/2012

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Abstract

Functional Expression, Ephrin Receptor Tropism, and Heterotypic Functionality of the Attachment and Fusion Glycoproteins of Cedar Virus, a Newly Discovered Henipavirus.

Stephanie R. Petzing

Thesis advisor: Dr. Christopher C. Broder, Department of Microbiology and Immunology, USUHS

The genus *Henipavirus* in the family *Paramyxoviridae* presently contains two members, Hendra virus (HeV) and Nipah virus (NiV), which are emerging zoonotic agents capable of causing serious disease in both humans and a variety of mammalian species. The reservoir hosts of henipaviruses are known to be several species of *Pteropus* fruit bats, and the mechanisms by which bats are able to tolerate infection with these highly pathogenic viruses are of great interest. With the recent isolation of *P. alecto* and *Rousettus aegyptiacus* cell lines, work is underway to identify potential bat cellular factors uniquely influencing viral dynamics. Serological surveys have detected antibodies to henipaviruses or henipa-like viruses in bats over a large geographic area, from the eastern coast of Australia to Ghana in West Africa. Despite this broad range of seropositivity in bats, HeV and NiV outbreaks have only been described in Australia, Malaysia, India, and Bangladesh, suggesting that bat populations might carry undiscovered viruses antigenically related to the henipaviruses. This has recently been confirmed by the discovery of Cedar virus (CedPV), the first new proposed member of the *Henipavirus* genus. Cedar virus was isolated from urine samples of flying foxes in

Australia and was shown to be genetically and antigenically related to HeV and NiV. CedPV also appeared to utilize the same entry receptor, ephrin-B2, that both HeV and NiV employ. The present work functionally characterizes the attachment (G) and fusion (F) glycoproteins of CedPV to provide greater detail on its receptor use and tropism, its relatedness to HeV and NiV, and aid in further defining the mechanisms and characteristics of *Henipavirus* mediated membrane fusion in general. It was found that the F glycoprotein of CedPV is synthesized as an F₀ precursor that is proteolytically cleaved into F₁ and F₂ subunits, and that CedPV glycoprotein mediated cell-cell fusion takes place at neutral pH and requires the presence of both the F and G glycoproteins. It was also determined that the envelope glycoproteins of CedPV are functional in an imperfect bidirectional heterotypic combination with the F and G glycoproteins of HeV and NiV. The combination of CedPV G expressed together with NiV F was fusion defective, whereas all other combinations of henipavirus G and F pairs retained the ability to mediate membrane fusion. Remarkably, it was also determined that CedPV G could bind not only the ephrin-B2 receptor protein, but also bound specifically to ephrin-B1 and the glycosphosphatidylinositol (GPI)-anchored ephrins-A1, -A2, and -A5. Further, the CedPV G binding activity to the majority of ephrins correlated with their use as functional receptors that could trigger CedPV glycoprotein mediated cell-cell fusion, imparting CedPV with a uniquely broader receptor tropism as compared to HeV and NiV. The present findings also have far-reaching implications for the study of *Henipavirus* ecology, particularly for understanding the potential of spillover into susceptible hosts. Further, these new data also provide a unique new tool set with which to study the

membrane fusion and virus entry mechanisms of the henipaviruses, potentially providing new avenues and strategies for the development of antiviral agents.

**Functional Expression, Ephrin Receptor Tropism, and
Heterotypic Functionality of the Attachment and Fusion
Glycoproteins of Cedar Virus, a Newly Discovered
Henipavirus.**

By

Stephanie Rachel Petzing

**Dissertation submitted to the faculty of the Department of Microbiology and
Immunology Graduate Program of the Uniformed Services University of the Health
Sciences**

F. Edward Herbert School of Medicine

**In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2012**

Acknowledgements

Thank you to Dr. Christopher Broder for excellent guidance and support, and thank you to my lab members and collaborators without whom this work would not have been possible. And thank you to Dr. Lee Metcalf, who believed in me from the very beginning.

Dedication

For my mother Ursula C. Petzing.

Thank you, mom, for always believing in me and supporting me and for fighting so hard to make sure that I would have the opportunities to pursue my passions.

Also for my family, Sebastian, Sonya, Chris, and Antony, thank you for your love and support.

And for my Oma and Opa Edzards and the forests of Germany, as well as Mr. and Mrs. General Petzing (Grandmother and Grandfather) and the Discovery Room at the Smithsonian Museum of Natural History.

Thank you all for instilling and fostering in me a love and appreciation of the natural world, a scientific curiosity, and a joy of discovery.

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Chapter 1

General Introduction

1.1 Hendra virus and Nipah virus discovery and outbreaks. Hendra virus and Nipah virus are two emerging zoonotic viruses in the family *Paramyxoviridae* which together make up the genus *Henipavirus* and are capable of causing lethal infections in humans. The natural reservoir host of both viruses have been shown to be bats, particularly *Pteropus* species flying foxes [1]. Hendra virus was first described following an outbreak of acute respiratory disease in 21 horses and two humans, in Queensland, Australia in 1994, causing the death one human and 14 horses [2]. It was initially described as an equine morbillivirus based on characteristic pleomorphic ultrastructural virion morphology, syncytia formation in the vascular endothelium of naturally and experimentally infected horses, a weak cross-reactivity with Rinderpest virus antisera, and due to a lack of hemagglutination or neuraminidase activity [2, 3]. Once sequence information became available it was found that Hendra virus was distinct from all previously described paramyxoviruses and so became the prototypic member of a new genus within the *Paramyxovirinae* [4].

HeV has continued to cause frequent outbreaks of disease in horses since 1995 and in recent years outbreaks have been recorded more frequently, to date totaling 35 spillover events. Horses serve as an intermediate and amplifying host of the virus, who can then infect humans, particularly those involved in husbandry and veterinary work, leading thus far to seven infections in humans, and causing four human fatalities (recently reviewed in [5]. A spillover event involving horses and the exposure of 11 humans was

confirmed in May 2012 and most recently in July 2012, a foal was confirmed to have fallen ill due to Hendra infection (http://www.daff.qld.gov.au/4790_2900.htm).

Nipah virus was described during an outbreak of encephalitis in Malaysia and Singapore in 1998-99 involving 276 human cases with 106 fatalities. Much like Hendra virus, an amplifying host, in this case domestic pigs, was required for the disease to spill over into the human population [6]. This led to the culling of close to 900,000 pigs and a ban on pig movement between farms, measures which brought the outbreak to an end [7], even as it caused a devastating amount of damage to the economy of Malaysia.

Following the initial outbreak, there have been at least 12 other nearly annual spillover events, primarily Bangladesh but also India, and there has been evidence of both food-borne and direct human-to-human transmission [8, 9]. The first outbreaks after the initial one in which NiV was discovered occurred in Bangladesh in 2001 and 2003. A retrospective study showed that risk factors included exposure to sick cows, and close contact with an infected person, indicating that person-to-person transmission was likely taking place [10]. This study also was the first to describe NiV antibodies in *P.*

giganteus fruit bats in Bangladesh. Another outbreak occurred between December 2004 and the end of January 2005 in Tangail District, Bangladesh. Of 12 affected persons, 11 died, leading to an alarming 92% fatality rate in this outbreak. This was the first outbreak in which a connection was made between the consumption of raw date palm sap and NiV infection. It was found that 64% of cases had consumed sap, while only 18% of controls reported this exposure. Upon interviewing date palm sap collectors it was found that fruit bats (*P. giganteus*) were considered nuisances which feed on the sap as it is collected overnight into pots hanging from date palm trunks under areas of denuded bark. They

also reported bat feces and even dead bats floating in the sap when the pots are collected early in the morning [11] shortly before being sold to consumers who drink it fresh. Since it is known that NiV can remain infectious for days in bat urine and in fruit juice [12], it is thought that NiV shed from the bats in saliva and urine entered the sap and subsequently infected the consumers, adding drinking freshly harvested date palm sap to the list of risk factors for NiV infection.

The high fatality rate (up to 78% in certain outbreaks) and the lack of vaccines or therapeutics have led the Centers for Disease Control to classify HeV and NiV as category C agents, restricting any work with live virus to biosafety level four (BSL-4) containment.

1.2 Lineages of henipaviruses. Nipah virus isolates from two outbreaks in Bangladesh in 2008 and 2010 were found to be significantly distinct from the NiV isolate obtained from the initial Malaysian outbreak in 1998-99 [13]. These three isolates were also significantly distinct from each other, even two that were obtained within three months of one another within a narrow geographical region, indicating that multiple lineages of NiV co-circulate temporally and geographically. These findings are in contrast to the data from the initial Malaysian outbreak, in which few differences in viral isolate sequence were observed. A NiV isolate obtained from *P. lylei* flying foxes was also found to be significantly different from both the Malaysia and the Bangladesh strains [14]. Overall there are presently three distinct lineages of NiV, NiV-Malaysia, NiV-Bangladesh, and NiV-Cambodia. Whether the higher case fatality rates and the ability to mediate person-to-person transmission observed in the India and Bangladesh outbreaks in comparison to

the apparent absence of NiV disease episodes in Cambodia are due to differences in the virulence of NiV lineages is not currently known.

1.3 Henipavirus biology. The genus *Henipavirus*, belonging to the subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales*, presently consists of two prototypical members, Hendra virus (HeV), and Nipah virus (NiV). HeV and NiV were determined to be molecularly similar enough to each other and distinct enough from other *Paramyxovirinae* [15, 16], that they were categorized into a new genus. The henipaviruses are pleomorphic, enveloped viruses with a non-segmented negative sense RNA genome and enter their target cells by pH-independent membrane fusion event that is facilitated by two membrane-anchored glycoproteins, the attachment (G) and fusion (F) glycoproteins. Despite having very large genomes, that, at 18.2 kb were considered the largest in the order *Mononegavirales*, until the characterization of Beilong virus and J virus [17, 18], the defining genome order typical of paramyxoviruses, 3'-nucleoprotein (N)- phosphoprotein (P)-matrix protein (M)-fusion glycoprotein (F)-attachment glycoprotein (G)-polymerase (L)-5', is conserved in the henipaviruses.

Replication – N, P, L complex. Like other paramyxoviruses, the genome of the henipaviruses conforms to the “rule of six”, meaning that each nucleoprotein subunit interacts with 6 nucleotides of the RNA genome to form a stable ribonucleoprotein complex. To maintain this interaction, the number of nucleotides in the genome of the paramyxoviruses must be divisible by six. The N protein of Nipah virus is 532 amino acids in length, with a molecular weight of approximately 60 kDa, and shares a ~78% homology [19] with the equally sized Hendra virus N protein [20]. It has been demonstrated that NiV N is phosphorylated at ser451, and that this phosphorylation is

critical for viral replication [21]. The N protein forms a complex with the P and L proteins which replicates the viral RNA both in the context of viral infection as well as *in vitro*. The functional importance of the henipavirus N/P/L complex in viral replication has also been demonstrated using a NiV minigenome system [22].

L protein. The HeV L protein is the viral RNA dependent RNA polymerase and is 2,244 amino acids in length with a molecular mass of 257 kDa [16], and the NiV L protein is identical in amino acid length and molecular mass. They share an 86.8% amino acid sequence identity and are rich in leucine and isoleucine residues.

P/V/C/W proteins. The P gene of HeV and NiV encodes a P protein of 707 and 709 amino acids, respectively, which are 67.6% identical at the amino acid level [4, 15]. The gene sequence of the henipavirus P gene contains a second open reading frame encoding the C protein, and the HeV C gene sequence (but not the NiV C ORF) additionally encodes a 65 amino acid small basic protein. The C genes of both HeV and NiV are 166 amino acids in length and share 83.2% sequence identity. The henipavirus P gene also contains an AG-rich editing site where additional, non-templated G residues are added to the mRNA, creating two additional open reading frames that encode a V protein by the addition of a single G residue, or a W protein when two G residues are added. The HeV V protein is 457 amino acids in length with a molecular mass of 50 kDa. The NiV V protein shares 81.1% amino acid identity with HeV V which is 454 amino acids in length. The W proteins of both HeV and NiV are 47 amino acids in length and share the amino terminal domain sequence with the V protein. Both the V and W proteins have been shown to antagonize the host cell interferon response by inhibiting the activation of interferon regulated genes by interferon β [23].

1.4 Matrix protein. The HeV M protein is identical in size to the NiV M protein and both being 352 amino acids in length, with a molecular mass of approximately 40 kDa and share 77.1% amino acid identity. As reviewed in [24], the M protein organizes virion structure and morphology and is the mediator of viral egress. The henipaviruses exit infected host cells by budding, and the matrix protein has been shown to be both necessary and sufficient for budding of both HeV and NiV [25, 26]. The M proteins are highly abundant in infected cells and interact with the attachment and fusion glycoproteins via their cytoplasmic tails and also interact with cellular membranes and the ribonucleoprotein core of the virus. Through these interactions they form the link between the viral genome, the envelope, and the F and G membrane glycoproteins. Additionally, some other paramyxovirus M proteins are known to mediate viral egress through their interactions with components of the host cell exocytic machinery via stretches of amino acid sequences termed late (L) domains because they act late in the virus replication and budding process. Two late domains have been identified in the NiV M: YMYL [26] and YPLGVG[27]. Mutation or deletion of these regions results in abrogation of budding and a nuclear retention phenotype.

1.5 F glycoprotein. The fusion glycoprotein (F) of the henipaviruses is a class I viral fusion protein which is synthesized as an inactive F_0 precursor which forms an oligomeric homotrimer. F_0 is cleaved by the host endosomal protease cathepsin L to form the F_1 and F_2 subunits which remain covalently linked via a disulfide bond [28], and it has been recently demonstrated that cathepsin B can also proteolytically cleave F in MDCK cells [29]. The mature henipavirus F glycoprotein homotrimer of cleaved F_1/F_2 monomers is similar to other paramyxovirus F glycoproteins [30, 31]. Henipavirus F

possesses multiple domains which are well conserved among other paramyxovirus fusion proteins, including a C-terminal cytoplasmic tail, a single helical transmembrane domain, two heptad repeat (HR) domains (A and B) (HRA and HRB), and a hydrophobic fusion peptide [32]. Mutational analysis of the TM domain of HeV F has suggested that the TM domain, and specifically its close location relative to the HRB, is critical for the regulation of F triggering by stabilizing the conformation of HRB and the TM domain-trimerization which facilitates the interactions between the monomers within the trimeric F oligomer and the stabilization of the metastable prefusion conformation of F [33].

1.6 G glycoprotein. The attachment glycoprotein of the henipaviruses is a type II membrane-anchored protein which binds to the virus entry receptors ephrin-B2 and ephrin-B3. Unlike the attachment glycoproteins of the majority of well-characterized paramyxoviruses which possess either a hemagglutinin (H) or hemagglutinin-neuraminidase (HN) attachment glycoprotein, the henipavirus G glycoprotein lacks both hemagglutination and neuraminidase activities. The primary sequence of HeV and NiV G possesses low level amino acid homology to other paramyxoviruses, with the greatest level of homology between HeV G and human parainfluenza virus 1 (hPIV1) being only 23% [20]. Despite the low level of primary sequence identity, the central features of paramyxovirus attachment glycoprotein secondary structures are conserved in the henipaviruses; including an N-terminal cytoplasmic tail, a helical transmembrane domain, a helical stalk region, conserved cysteine residues mediating inter-and intramolecular disulfide bonds, and a globular head [15, 34, 35]. The globular head of G assumes a 6-bladed (B1-B6) β -propeller conformation, with each blade of the propeller consisting of four or five β sheets in an antiparallel arrangement connected by loops or helices [36]. In

the center of the propeller is a funnel-shaped cavity where receptor binding takes place. The mature form of the G glycoprotein is a tetramer consisting of a dimer of disulfide-linked dimers [37] The structures of both the HeV and NiV G glycoprotein head domain alone and in complex with ephrin receptors have also been determined revealing the molecular details and specificity of the virus attachment process, discussed below.

1.7 G Interactions between henipavirus G glycoproteins and the ephrin receptors.

Both HeV and NiV have been shown to enter host cells using either ephrin-B2 or ephrin-B3 as receptors, and through its interaction with receptor, the G glycoprotein is the main determinant of viral cell and species tropism [38-40]. The ephrin family of proteins is known as membrane-anchored ligands consisting of two subfamilies, the glycosylphosphatidylinositol-anchored A-type ephrins, and the transmembrane anchored B-type ephrins. Together with their partner cellular receptors the eph proteins, these molecules mediate bi-directional signaling events throughout the body and play a role in numerous cellular processes, including neurogenesis, cortical development, formation of vasculature and cancer [41-45]. Ephrin-B2 and ephrin-B3 are receptor tyrosine kinases which are highly conserved across the animal genera, and it has been shown that recombinant expressed ephrin-B2 and ephrin-B3 molecules of human, horse, pig, cat, dog, bat and mice can serve as entry receptors for both HeV and NiV [46]. This accounts for the remarkably broad species tropisms of HeV and NiV, and differentiates them from the other members of the Paramyxoviridae family which as a group tend to have a much greater restricted host range. The expression patterns of ephrin-B2 and -B3 *in vivo* also correlate well with the clinical manifestations of henipavirus pathogenesis. Ephrin-B2 is expressed in arterial smooth muscle cells, placental tissue, the spleen, lymph nodes,

neurons, and endothelial cells, while ephrin-B3 is more prominent in the nervous system, vasculature and lymphoid cells [47, 48]. This ephrin receptor distribution pattern also accounts for the observed vascular endothelial tropism and pathology observed in henipavirus disease [49] as reviewed in [50].

The interaction between G and ephrin-B2 and -B3 has been mapped to the same area which mediates the interaction between ephrin-B2 and -B3 and their cognate ephrin receptors [51], an 18 residue domain called the G-H loop, and particularly to a six residue subdomain, $_{120}\text{FSPNLW}_{126}$ [52]. The restriction of the henipavirus G to ephrin-B2 and -B3 despite high levels of sequence and structural conservation among the different ephrins has been postulated to be due to the structure of the G-H loops. The GPI-anchored A class ephrins differ in sequence and structure of the G-H loop, and it is thought that they would not be able to fit into the binding cavity of G [36]. The only remaining B-class ephrin, ephrin-B1, differs in the sequence of the G-H loop primarily by the presence of the Tyr124 and M125 residues in place of the $_{124}\text{LW}_{125}$, which are thought to cause steric hindrance, preventing binding of G. In support of this hypothesis, site directed mutagenesis studies converting the G-H loop sequence of ephrin-B1 to that of ephrin-B2 were carried out, and it was found that upon mutation of those two amino acids, NiV glycoproteins were able to mediate fusion with cells expressing the mutated ephrin-B2 [39].

The crystal structures of henipavirus G glycoproteins, both alone and in complex with receptor, have been determined [52-54], and are very similar among the three solved possible complexes (NiV G/B2, NiV G/B3, HeV G/B2). Interestingly, the conformational change between free and receptor bound G is minimal and mostly

restricted to the loops which directly contact ephrin [36, 54]. This was unsurprising given the fairly rigid structure of the globular head of G, a 6-bladed β -propeller which is stabilized by an extensive network of inter- and intra-blade hydrogen bonds, Van Der Waals forces, as well as disulfide bonds [52, 53]. This observation indicates that the triggering of F by G is not simply due to large conformational changes of the monomeric glycoproteins, and likely depends on higher order changes of the tetrameric oligomers.

Ephrin-B2 and ephrin-B3 share ~40% amino acid identity and whose overall structure is very rigid, with the exception of the fairly flexible G-H loop [51]. Upon G engagement, the conformational changes of the ephrin molecules are mostly restricted to this G-H loop, particularly the four hydrophobic residues at the tip (Trp125, Leu124, Pro122, Tyr/Phe120) which are tightly fitted into four pockets in the receptor binding central cavity of G. Additional sites of ephrin-G interaction are F120 of ephrin-B2 which inserts into another pocket in the binding cavity of NiV G, and Glu533 in NiV G which has been shown to form salt bridges with Arg57 and Lys116 of ephrin (reviewed in reference [36]).

1.8 Henipavirus mediated membrane fusion. The henipavirus attachment (G) and fusion (F) glycoproteins are expressed as membrane-anchored proteins on the surface of virions as well as on infected host cells. Both the G glycoprotein tetramer and the F glycoprotein trimer are required for facilitating the membrane fusion process (reviewed in [55, 56]). Once the attachment glycoprotein engages receptor, it undergoes an as yet poorly defined conformational change which triggers F to unfold and extend [57], exposing the hydrophobic fusion peptide, which inserts into the target cell membrane.

The conformation of the F glycoprotein then progresses through further major structural rearrangements, folding back on itself forming a six-helix bundle (6HB) structure which brings the membranes of the target cell and the virion into close proximity and the formation of the 6HB appears to occur concomitantly with lipid mixing, and hemifusion, which is followed by fusion pore formation membrane merger and entry of the viral nucleocapsid into the target cell cytoplasm (reviewed in [55, 58]J. White or R. Dutch or others).

Much work has focused on elucidating the structural rearrangements of F that follow receptor engagement by G and membrane merger process. Crystal structures of other paramyxovirus F glycoproteins have revealed dramatically different prefusion (PIV5) [59] and post-fusion (hPIV3) [60] conformations of F. Prior to receptor binding by an attachment glycoprotein, the mature cleaved F is expressed on the surface of the virion in a metastable prefusion state. The F glycoprotein consists of a trimeric coiled coil stalk region made up of the HRB sequences of the three monomers, topped by a globular head with three domains (DI, DII, DIII) [59]. DIII provides a scaffold which prevents HRA from folding into HRB and assuming a post-fusion structure. In the trimer N-terminal hydrophobic fusion peptides solvent protected by being hidden in the subunit interfaces of adjacent monomers [59]. In the post-fusion state, the F glycoprotein adopts a more energetically favorable “hairpin” conformation characterized by the residues of HRB aligning into the grooves of HRA, folding the F glycoprotein back over itself and forming a “6- helix bundle” (6HB) [60]. In between the pre- and post-fusion states, the F glycoprotein exists in an extended “pre-hairpin” form in which the fusion peptide is extended toward the target membrane by an opening of the F structure into an extended

parallel coiled coil [57]. This opened structure allows peptides homologous to the HRB helix to access the HRA helix, binding and occluding that site, causing fusion to be inhibited by preventing 6HB formation [61, 62].

While an understanding of the paramyxovirus fusion process has increased greatly in the last decade, there still remain several unanswered questions. The question of the nature and timing of the interactions between paramyxovirus F and G glycoproteins are the subject of much research and debate (recently reviewed in [63]) which has centered primarily on a “clamp” versus “provocateur” models of fusion. The “clamp” model suggests that the fusion glycoprotein is maintained in a prefusion state by constant interaction with the G glycoprotein. Once the G glycoprotein engages receptor, G dissociates from F, relieving the restriction and allowing F to undergo conformational changes required to mediate fusion. This model presupposes that F without G would assume a postfusion conformation, requiring that the glycoproteins co-traffic through the endoplasmic reticulum and remain associated with each other when they reach the cell surface, preventing premature triggering of F by constant association with G. Whereas the “provocateur” model suggests that the role of the G glycoprotein in triggering fusion is actually more active than simply preventing any premature conformational change or triggering of F. Rather, it suggests that upon receptor binding, G undergoes conformational changes which then promote its association with F leading to the triggering of its fusigenic activity. The details of the fusion triggering process remain obscure and may involve F and G dissociation, F and G association, or perhaps only receptor-induced conformational changes in either the monomers comprising the

tetrameric G or changes in the association of dimers within the native G tetrameric oligomer.

Current studies on henipavirus fusion mechanisms seem to partially support both models. It is known that henipavirus F and G interact with each other without any requirements for receptor engagement, as the F and G glycoproteins can be co-immunoprecipitated in the absence of receptor [64]. It is not known exactly how the F and G glycoproteins associate with each other, but isoleucine residues in a helical region of the stalk domain of G have been implicated [35], as well as residues in the globular head of F. How this interaction prevents premature triggering of F is unclear, but it seems likely that this is the case, as the conformational changes following triggering are irreversible and would therefore need to be timed with the presence of an appropriate host cell membrane in adequate proximity to F to initiate fusion. The residues in the stalk of HeV G which are critical for interaction with F are located in a structurally conserved alpha helical region that is implicated in tetramer formation. Mutation of these residues not only rendered G incapable of interacting with F, but also enhanced the binding profile of mutant G with certain monoclonal antibodies which preferentially bind a G/receptor complex, indicating that the mutations resulted in G adopting a receptor-bound conformation in the absence of receptor [35]. These results suggest that not only must G interact with F in a pre-receptor bound form, but that the presence of a receptor-bound form of G is not sufficient to trigger F glycoprotein mediated fusion.

Taken together, these data seem to support a “clamp” mediated fusion mechanism for henipaviruses, by which the F glycoprotein is prevented from the required conformational changes to mediate fusion until triggered to do so by receptor engagement

of the G glycoprotein. However, the clamp model is not supported by the observation that Co-trafficking of the F and G glycoproteins of HeV and NiV has not been observed [65], and it is known that the F glycoprotein undergoes a unique endosomal recycling step where, after trafficking to the cell surface, the F₀ glycoprotein is transported back to an endosomal compartment where it is cleaved into the biologically active F₁ and F₂ forms by cathepsin L and subsequently restored to the cell surface [66, 67], essentially precluding the possibility of a significant intercellular pre-association of F and G. Additionally, recent work employing cholesterol tagged peptide inhibitors of fusion has indicated that the G glycoprotein must continue to interact with F throughout the process, even after the FP has been inserted into the target membrane. This support a “provocateur” model by expanding the role of the paramyxovirus G glycoprotein in regulating the conformational changes of F from a solely inhibitory role to an active chaperone of the stepwise transitions of F structure from the metastable prefusion state, to the extended prehairpin intermediate, to the insertion of the fusion peptide, to 6HB formation and membrane merger [68].

As more data is gathered on the molecular details of the paramyxovirus fusion process, it seems that the “provocateur” model may be most accurate. The question remains however, how does G “provoke” F to undergo fusion? It has recently been shown that the higher-order oligomeric state of the attachment glycoprotein may be involved in the regulation of F mediated fusion, as a mutation in the dimer-dimer interface of HPIV3 HN has been shown to enhance F activation and increase fusion in cell culture experiments [68]. Interestingly, HPIV3 with the mutated HN demonstrates reduced fitness in a cotton rat model, testifying to the close regulation of fusion which is

required for successful viral replication [69]. Recent work on the stalk of morbillivirus attachment glycoprotein (H) has lent further support to the role of the tetrameric state of the attachment glycoprotein in triggering F. Intermolecular disulfide bonds were engineered into the stalk domain of H, in a region in which overlaps with a putative F interacting domain. These mutants resulted in inter-subunit cross-links and abrogated membrane fusion. The fusogenic activity of H was restored when fusion was carried out under reducing conditions. These data suggest that the disulfide linkage prevented the oligomeric H from undergoing conformational changes required to activate F, although the nature of these conformational changes remains unknown [70].

While more work is needed to reconcile the seemingly conflicting data into one complete model of henipavirus fusion, the data seems to support the following model of henipavirus fusion: The attachment and fusion glycoproteins are synthesized and traffic separately to the cell surface. The F glycoprotein then undergoes retrograde transport from the cell surface to a recycling endosome where the inactive F_0 precursor is cleaved to the active F_1/F_2 form of the glycoprotein by the endosomal protease cathepsin L. The mature, cleaved F_1/F_2 glycoprotein is then transported back to the cell surface. The G glycoprotein is present as a tetrameric dimer of dimers which interacts with and stabilizes the homotrimeric F glycoprotein in an incompletely characterized manner involving residues in the stalk of G and the globular head of F. Following receptor binding, the G glycoprotein tetramer undergoes conformational changes which may involve the dissociation of the dimers pairs. These changes are necessary, but perhaps not sufficient, to trigger F. Once triggered, F undergoes a series of conformational changes which require continuous receptor engagement by G. First, the coiled coils of the stalk melt and

the F glycoprotein assumes an open extended form (the pre-hairpin intermediate), stretching the fusion peptide toward the host cell membrane. Then the fusion peptide inserts into the membrane, and the alpha helices of HRB fold tightly into the grooves of HRA, forming the 6HB which folds the glycoprotein back onto itself into a post-fusion hairpin conformation. This process forces the host membrane into close enough contact with the membrane of the virion that lipid mixing occurs, which leads to hemifusion, pore formation, and finally entry of the virion nucleocapsid into the host cell (recently reviewed in [71]).

Despite many recent advances in our understanding of the *Henipavirus* mediated membrane fusion mechanism, the complex nature of the interaction between F and G, as well as the mechanism by which F is triggered remain active areas of investigation. A greater understanding of these processes could lead to the rational design of drug targets designed to interrupt the cycle of infection.

1.9 Henipavirus disease. Details of the clinical disease process and progression

following HeV or NiV infection has been recently reviewed in [72], and are overall quite similar to one another. The incubation period is generally short and initial symptoms present as a generalized “flu-like” illness which can progress to pneumonia or acute encephalitis leading to death. It is thought that infection of neurons occurs subsequent to infection and destruction of vascular endothelial cells leading to a compromise of the blood-brain barrier. In some cases of either HeV and NiV infection, patients that recover from the acute illness can later suffer a recrudescence of virus replication resulting in relapse encephalitis that can occur from several months to years later, in one case relapse encephalitis occurred 11 years following an initial NiV infection [73]. The overall

mortality rate of HeV infection is 50%. The human case mortality rate of NiV infections ranges between 40% and 100%, depending on the outbreak (reviewed in [74, 75]. It is not known what factors contributed to the increased mortality rates of NiV infection in the outbreaks in Bangladesh, and while human factors such as access to medical care have likely played a role, the contribution of strain differences in the infecting virus, as discussed earlier, cannot be discounted and are being explored.

1.10 Animal models. In order to explore the pathogenesis of the henipaviruses as well as establishing platforms for the testing of vaccines and therapeutics, several animal models have been established (reviewed in [76]). Initial experimental animal infections with HeV were carried out in horses, which developed a primarily respiratory disease. A variety of other animal species were later examined including guinea pigs, cats, mice, rats, chickens, rabbits and dogs. Among these, only cats and guinea pigs developed HeV disease upon challenge, characterized by respiratory distress with high fatality rates., Upon necropsy, these animals displayed gross lesions of pneumonia which were histologically similar to the lesions found in horses that succumbed to HeV infection.

The first experimental animal infections with NiV were carried out in pigs, bats and cats. Infection of pigs with NiV caused both respiratory and nervous system disease sometimes leading to death, as well as shedding of virus by infected pigs without clinical signs of disease. Experimentally infected cats displayed a similar disease following challenge but the disease was characteristically more severe, and at necropsy both the cats and pigs showed evidence of systemic vasculitis, alveolitis, and meningitis. Experimental infection of pteropid bats with HeV or NiV has failed to cause any detectable illness, but does often result in seroconversion, an outcome considered to be

expected as they are the natural hosts of both these viruses [1]. More recently, golden hamsters, ferrets and African green monkeys have been explored and each have been shown to accurately recapitulate the human disease manifestations observed as a result of either HeV or NiV infection (reviewed in [76]. The guinea pig model is regarded as somewhat unsuitable for use in the evaluation of vaccines or therapeutics due to inconsistent disease outcomes and a low incidence of encephalitis. The pig and horse models, while challenging to work with in a BSL-4 setting, are good models of infection and disease in these epidemiologically relevant hosts and are valuable for studying transmission to humans and for evaluating livestock vaccines designed to interrupt the cycle of transmission.

1.11 Henipaviruses and bats. The natural reservoir hosts of both HeV and NiV have been demonstrated to be fruit bats [1] of the *Pteropid* genus, although serological evidence indicates that the natural reservoir host range of henipaviruses may extend to non-pteropid fruit bats and even some insectivorous bats [77-79]. The first detection of HeV neutralizing antibodies in bats were described in all four species of fruit-eating bats (flying foxes) in eastern Queensland, *Pteropus alecto*, *P. scapulatus*, *P. conspicillatus*, and *P. poliocephalus*, in 1996 [80]. Not long thereafter, Halpin and colleagues [81] reported the isolation of three HeV isolates from the uterine fluid and fetal tissues of a *P. poliocephalus* grey-headed flying fox, and from the fetal lung of one *P. alecto* black flying-fox, clearly demonstrating that these species of fruit bats are environmental reservoirs of HeV.

When NiV emerged and was characterized in 1998-99, it was observed that the pigs which served as amplifying hosts of the virus in the initial outbreak were kept in areas

that were directly accessible by fruit bats, which, along with the clear relatedness of NiV to HeV, led to the undertaking of serological studies of nearby bats. Neutralizing antibodies to NiV were found in 5 different bat species: the insectivorous *Scotophilus kuhli*, the non-pteropid frugivorous bats *Eonycteris spelaea* and *Cynopterus brachyotis*, and two pteropid frugivorous bats, the Island flying fox (*P. hypomelanus*) and the Malayan flying fox (*P. vampyrus*) [82]. Subsequently, following the collection of urine from a *P. hypomelanus* colony, and partially eaten fruit discarded by these bats, located off the coast of Malaysia on Tioman island, an isolate of NiV was obtained which was nearly identical to the sequence of the human isolates obtained in the first outbreak [83]. Outbreaks of NiV in Bangladesh in 2001 led to a serosurvey of the local flying fox population, and neutralizing antibodies to NiV were obtained from *P. giganteus* [10], a species of flying fox whose flight range extends across the Indian subcontinent [84]. While neutralizing antibodies to NiV in *P. giganteus* in Bangladesh have been found during two subsequent serological studies [85], including a remarkable 50% positive rate in a single colony in northern India, to date no NiV isolate from bats in India or Bangladesh has been reported, though human isolates are available.

The detection of henipavirus reactive antibodies or RNA has not been limited to countries where outbreaks have occurred. In Thailand, IgG to NiV was found in the serum of three *Pteropus* fruit bat species and one *H. larvatus* insectivorous bat. Additionally, NiV RNA was found in the saliva of *P. lylei* and *H. larvatus*, as well as in the urine of *P. lylei* [86]. In addition, evidence of henipavirus infection was found in two species of fruit bats, *Eidolon dupreanum*, and *P. rufus* on the island of Madagascar. Interestingly, of the 20 bats which were positive for henipavirus reactive antibodies by

ELISA, only 3 were found to carry neutralizing antibodies to HeV and the Malaysian strain of NiV, and 1 was found to neutralize only NiV. This suggests that the circulating henipaviruses in Madagascar are clearly antigenically distinct from those circulating elsewhere, however since no isolate was obtained, the details of these differences are unknown. Additionally, the presence of henipavirus infection in *Eidolon* species fruit bats implies that henipaviruses might also be present in Africa, the only other location where bats of this genus are found [87].

Recently, antibodies reactive to both HeV and NiV were found in *E. helvum* fruit bats in Ghana, over 5,000 km away from the nearest *Pteropus* species of bats [88], demonstrating that the distribution of henipa-like viruses is not limited to the distribution of pteropid flying fox species as was previously thought. Another survey of *E. helvum* fruit bats in West Africa discovered *henipavirus*-related RNA in fecal samples from a colony located in an urban setting in Ghana. These sequences were phylogenetically clustered with pteropid bat isolates of HeV and NiV, but were divergent enough that they suggested the presence of additional, as yet uncharacterized strain of henipaviruses in Africa. Unfortunately the RNA concentrations were too low to obtain complete sequence data and no viral isolate has been obtained [89]. In addition, a serological study in domestic pigs was carried out in Ghana to determine if a transmission cycle of bat to pig was possible as had occurred in the initial outbreak and discovery of NiV in Malaysia. Non-neutralizing antibodies to HeV and NiV were found in a population of domestic pigs residing near the roosting grounds of a large colony of *E. helvum* fruit bats. These data further emphasize the likely presence of novel henipa-like viruses in Africa, and that the potential for zoonotic transmission to humans analogous to the initial NiV outbreaks in

Malaysia is possible [90]. Some 340km off the Western coast of Africa, in the Gulf of Guinea lays the small island of Annobon. Being small and remote, Annobon supports a population of *E. helvum* fruit bats that has been in isolation for so long that they have developed both morphological and genetic differences significant enough to designate them as a subspecies *E. helvum annobonensis* [77]. Due to their isolation and small size, it was thought that this population would be unable to support endemic infection by henipaviruses, however, a serological survey demonstrated henipavirus reactive antibodies in this population [77], again expanding the recognized range of the henipaviruses. These data also suggest that a mechanism of henipavirus maintenance within a population of bats may be due to infection followed by recrudescence, since new virus introduction events into a bat population so remote seems unlikely [77].

Since these initial serological studies, several groups have also conducted additional serosurveillance experiments seeking evidence of henipavirus infection in various bat species in a wide variety of locations. To date, henipavirus reactive antibodies have been described in thirteen different countries, and across nine species of pteropid flying foxes, six species of non-pteropid fruit bats, two species of microbat, and an insectivorous bat (recently reviewed in [91]).

This remarkably wide distribution of henipaviruses over 17 different species of bat and in thirteen countries indicates that the potential for spillover events and the number of at-risk people and/or livestock is significant. There are a number of environmental risk factors for henipavirus infection, such as close contact with infected domestic animals. Further, there are social and cultural practices such as the consumption of bats for food, or the drinking of bat blood [92], which may provide a risk of infection. *P.rufus*, one of

three fruit bat species in Madagascar is listed as “vulnerable” on the International Union for Conservation of Nature (IUCN) redlist of endangered species, which lists a 30% population decline in the last 20 years and a continuing downward trend in population numbers of a particular species. This is due partially to a loss of trees to agricultural development, and partially due to killing of bats, both to protect economically valuable fruit crops from being eaten or damaged and for consumption (<http://www.iucnredlist.org/details/18756/0>). With the likely presence of *henipavirus* infection in *P. rufus* demonstrated by serology, it seems likely that the butchering and consumption of *P. rufus* in Madagascar could represent a significant risk factor for the acquisition of henipavirus infection in this human population.

Additionally, the presence of antibodies reactive to henipaviruses in locations where no outbreaks have occurred and no isolates of HeV or NiV have been found, strongly suggests the presence of related henipa-like viruses in other bat populations. Until recently however, the absence of a virus isolate antigenically related to, but distinct from, HeV and NiV, has made the existence of such related viruses only a speculation.

1.12 Overview of Specific Aims.

Specific Aim 1.1: Determine if the basic elements of henipavirus entry into and egress from the cells of their natural reservoir hosts are different in comparison to other susceptible mammalian cells using viral glycoprotein mediated cell-cell fusion and virus-like particle budding assays.

Specific Aim 1.2: Clone the bat homolog of human AP3B1, a protein demonstrated to inhibit NiV matrix mediated budding of virus-like particles by binding to M (W. Sun and A.P. Schmitt, manuscript in preparation).

Specific Aim 2: Functionally express and characterize recombinant CedPV F and G glycoproteins and define the requirements of CedPV glycoprotein mediated cell-cell fusion.

Specific Aim 3: Determine if the F and G glycoproteins of CedPV exhibit heterotypic functional capacity when co-expressed with the F and G glycoproteins of HeV and NiV.

Chapter 2

Cellular factors mediating viral tolerance in bats

2.1 Introduction. Bats are increasingly being recognized as bearers of significant viral diseases, harboring numerous and deadly viruses of varied types. With over 1200 species, bats are second only to the order Rodentia in species variety and abundance, making up over 20% of the mammalian species diversity on earth [93]. Bats have a number of characteristics which make them unique from other mammals and may contribute to their ability to support a large and diverse population of viruses (reviewed in [94]). They tend to live in exceptionally large groups, are long-lived, with some species of insectivorous bats living as long as 25-35 years, and are the only mammals capable of true flight, allowing them access to a broad geographical area, in cases of migrating bats, this can be hundreds of miles. These traits may allow bats to sustain a large number of viruses with great diversity at a population level. At an individual level, a long period of co-evolution with infectious agents has likely allowed the chiropteran immune system and physiology to control infection in ways that are not yet understood. The extreme drop in body temperature and metabolic rate during short periods of daily torpor, or long periods of torpor during hibernation, may represent one mechanism by which the unique properties of bat physiology control viral infections.

With the recent identification of bats as likely reservoir hosts of many viruses of significant risk to the health of humans and livestock, including the high profile and highly dangerous Ebola and Marburg viruses of the *Filovirus* family there has been a resurgence of research investigating bats and their relationships to viruses and human health. This represents a renewed interest in work that was begun with the discovery of

rabies virus in bats in the 1920s, and which was periodically intensified over the next 60 years as bats were discovered to play a significant role in the ecology of other viruses such as Japanese encephalitis virus [95]. Over the course of this period, a number of studies were carried out investigating the nature of the chiropteran immune responses to viral infection in an experimental setting, experiments which became prohibitive as the funding priorities changed, and as much needed laws and regulations governing not only infectious agents, but also wildlife and endangered species were developed and came into effect. Although the results of these studies can be difficult to find and interpret due to changes in terminology over the years, they ought not to be overlooked in our current approaches. A re-interpretation of these reports, through the lens of advances in knowledge of the immune system can be useful. Significantly, many reports of experimental infection of bats with infectious agents seem to agree that the humoral immune responses of Chiroptera tend to be rapid and broad, but that the antibodies are of relatively low affinity, and that this response is short-lived. Given that this might indicate that bats are less likely to mount a strong inflammatory immune response following infection, these results might be relevant to the study of henipaviruses and filoviruses in bats, especially because fatal infection with these virus genera tends to involve a strong host inflammatory response.

Several theories attempting to explain the relationship between bats and viruses were recently reviewed by Wang and colleagues [94], including the supposition that the sheer species diversity and biomass of bats can account for the number and diversity of viruses they seem to host; that the ecological characteristics of bats allowed them to survive the most recent mass extinction event (the K-T extinction, 65 million years ago;

also known as the Cretaceous–Tertiary extinction event [94]) in high enough numbers, as well as geographical and ecological diversity making them rather uniquely situated as hosts of diverse viruses. Also, that bats, being of great aforementioned ecological and geographical diversity, and existing in great numbers and being largely evolutionarily unchanged since the major family diversification of Chiroptera approximately 62 million years ago, have had a long period of co-evolution with various viruses. This extended period has allowed for a fine-tuned interplay between the infecting viruses and the immune systems of the bats which host them, facilitating the survival of both the parasite and host.. These various theories are not mutually exclusive, and surely future research combining the fields of molecular virology, immunology, bat ecology, evolution and others will demonstrate that all these factors, as well as others yet to be considered, play a role in the complex interactions between viruses and their chiropteran hosts.

Recently, the first cell lines derived from *P. alecto* and *R. aegyptiacus* fruit bats, both relevant to henipavirus biology have been developed, offering a new opportunity to examine the role of bat cellular biology in viral dynamics. Two cell lines were isolated from *R. aegyptiacus* fetal tissues, RO6E from a fetal body, and RO5T from a fetal head. Unfortunately, the RO6E cell line was later found to be contaminated with African green monkey kidney (Vero E6) cells, and while the cell line has since been re-cloned and verified [96], the new RO6EJ cell line is not included in the work presented here. Several cell lines were isolated from *P. alecto* tissue including lung, kidney, brain, and two from fetal tissues [97], and these lines are the main focus of this section. Since very little is known about the cell biology of bats, it is possible that there are as yet undiscovered cellular mediators of viral control that are uniquely at play in bat cells. Whether these are

known molecules with mechanisms exhibiting a different functionality in bat cells, or entirely new molecules and cellular processes is not yet known.

2.2 Aims and Hypotheses

My overarching hypothesis is that viral tolerance in bats may be linked to bat-specific cellular factors influencing viral replication. I addressed this through the following aims:

Specific Aim 1: Determine if the basic elements of henipavirus entry into and egress from the cells of their natural reservoir hosts are different in comparison to other susceptible mammalian cells using viral glycoprotein mediated cell-cell fusion and virus-like particle budding assays.

Hypothesis 1: A viral glycoprotein-mediated cell-cell fusion assay can be employed to study the fusion characteristics of henipavirus glycoproteins with cells derived from their natural reservoir host.

Hypothesis 2: Henipavirus particle assembly and budding in cells derived from their natural reservoir host can be studied using a viral M protein driven virus-like particle (VLP) budding assay.

Specific Aim 2: Clone the bat homolog of human AP3B1, a protein demonstrated to inhibit NiV matrix mediated budding of virus-like particles by binding to M (W. Sun and A.P. Schmitt, manuscript in preparation).

Hypothesis: Significant sequence differences in the bat homolog of AP3B1 as compared to the human protein will be found and these differences may regulate henipavirus particle assembly and budding.

2.3 Materials and Methods

Cell lines. 293T cells were provided by G. Quinnan (USUHS). Vero cells were a provided by A. O'Brien (USUHS, Bethesda, MD) and were maintained in DMEM with 10% serum, L-glutamine, Pencillin/Streptomycin. The *R. aegyptiacus* cell line RO5T was derived from the “head” of a fetal bat and was maintained in DMEM/F12 with 5% serum [98]. The *P. alecto* cell lines PaKiT (kidney), PaFeT (fetus), PaLuT (lung), PaBrT, and PaBrH were kind gifts of Dr. L-F Wang (Australian Animal Health Laboratory (AAHL), of the Commonwealth Scientific and Industrial Research Organisation (CSIRO) , Geelong, Australia) [99], and were maintained in DMEM with 10% serum, L-glutamine, Pencillin/Streptomycin. All cell lines were kept at 37°C and 5% CO₂.

HeV glycoprotein mediated cell-cell fusion. Viral glycoprotein-mediated cell-cell fusion assays were carried out as previously described [100, 101]. Effector HeLa-USU cells in T-25 flasks were co-transfected with 750 ng of HeV F encoding plasmid and 2250 ng of a HeV G encoding plasmid giving a 1:3 ratio of viral F and G genes and a total of 3µg of DNA using Fugene® 6 transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany). 293T, RO5T, PaKiT, PaFeT, PaLuT, PaBrT, and PaBrH target cells were seeded at a density of 8×10^5 in T-25 flasks. Following an overnight incubation at 37°C, the effector cells were infected with recombinant vaccinia virus vTF7.3 at a multiplicity of infection (MOI) of 10 encoding the bacteriophage T7 RNA polymerase, and the target cells were infected with recombinant vaccinia virus vCB21R (MOI 10) containing the *E.coli lacZ* reporter gene under a T7 promoter. After a 3 hr incubation at 37°C, remaining extracellular virus was removed and the cells were transferred to conical tubes and incubated overnight at 31°C to reduce vaccinia virus mediated cytopathology.

The next day the cell populations were washed and counted and resuspended at a density of 1×10^6 cells/ml in DMEM-10 containing cytosine arabinoside (40 μ g/ml) to inhibit vaccinia virus superinfection and early gene transcription. 100 μ l of each cell population (1:1 ratio) were mixed in a 96-well plate and fusion was allowed to proceed for 2.5 hours at 37°C. Effector-target cell fusion resulted in cytoplasmic content mixing, allowing the T7 polymerase in the effector cell cytoplasm to mediate production of β -galactosidase from the lacZ gene cassette in the cytoplasm of the target cells. Following fusion the cells were lysed at room temperature by adding Nonidet P-40 alternative to the plate at (0.5% final), followed by one freeze-thaw cycle at -80°C. The lysates were thawed and mixed, and a 50 μ l aliquot was transferred to a new flat bottom 96-well plate. 50 μ l of 2X chlorophenol red-D-galactopyranoside (CPRG; Roche Applied Science, Indianapolis, IN.) was added to each well, and viral fusion rates were determined by the cleavage of the colorimetric substrate by β -galactosidase over time which was read on a VersaMAX microplate reader (Molecular Devices, Sunnyvale, CA) at room temperature at an absorbance of 570 nm. RO5T and Vero target cells were either unmodified, or transfected with an ephrin-B2 encoding plasmid, or infected with recombinant vaccinia virus vMB2 encoding ephrin-B2.

Virus-like particle (VLP) budding assay. The VLP budding assay was carried out essentially as previously described [25, 102, 103]. 293T or PaKiT cells were seeded in 10cm dishes which had been pre-coated with 100 μ g/ml of poly-D-lysine (MP Biomedicals LLC, Solon, OH) to enhance cell attachment, and transfected with 2 μ g of expression plasmids NiV matrix, CedPV matrix, or an empty vector, using LipofectamineTM and PlusTM Reagent at a 1:6:1 ratio of DNA:LipofectamineTM: PlusTM

Reagent. Following an overnight incubation, the cells were radiolabeled with 150 μ Ci/dish of 35 S labeled methionine and cysteine. 18 hours after applying radiolabel, the cells were harvested for immunoprecipitation with rabbit anti-NiV M polyclonal sera (A.P. Schmitt, Penn State University), or using S-agarose beads (EMD Biosciences Inc., San Diego, CA) and supernatants were collected for VLP preparation. Cells were pelleted by centrifugation and lysed in lysis buffer (100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1% Triton X-100). Lysates were immunoprecipitated using S-protein agarose beads (EMD Biosciences Inc., Madison, WI). Supernatants were clarified by centrifugation at 8,000 rpm for 2 minutes and overlaid into a cushion of 4ml 20% sucrose in 1X TEN buffer (.1M NaCl/10 mM Trizma base/1 mM EDTA) prepared in Ti70.1 polycarbonate centrifuge bottles (Beckman Coulter, Inc., Brea, CA). VLPs were pelleted by ultracentrifugation at 40,000 rpm for 1.5 hours at 4°C using a Ti70.1 swinging bucket rotor (Beckman Coulter, Inc.). The sucrose cushion and remaining supernatant was aspirated and the VLP pellet was resuspended in 0.9 ml of 1X PBS (Quality Biological Inc.). 2.4 ml of 80% sucrose in 1X TEN was added to each tube and mixed well to form an approximately 60% sucrose solution. Then a discontinuous gradient was formed by overlaying 3.6ml of 50% sucrose followed by 0.6ml of 10% sucrose. The VLPs were floated through this gradient by ultracentrifugation at 40,000 rpm for 3 hours at 4°C. Three 1.5 ml fractions were collected from the top of the gradient using Auto Densi-Flo gradient fractionator (Labconco, Kansas City, MO) and were mixed with 12 ml of 1X TEN in polycarbonate Ti50.2 tubes (Beckam Coulter Inc.). The VLPs were pelleted once more by ultracentrifugation at 40,000 rpm for 1.5 hours at 4°C. The buffer was aspirated and the VLP pellets were resuspended in SDS-PAGE

sample buffer containing 2-mercaptoethanol , boiled for 5 minutes, and run on an 14x15 cm SDS-PAGE gel (Hoefer Inc., San Francisco, CA) at 20V for 14 hours along with the corresponding cell lysates. The SDS-PAGE gel was dried in a vacuum manifold for 2.5 hours at 80°C and placed on film for autoradiography.

Plasmids. pCAGGS-eGFP contains an enhanced green fluorescent protein (eGFP) gene under the control of an hCMV promoter and was a gift of D. Weir (USUHS). pMX-GFP, an MLV-GFP vector was a kind gift of Dr. E. Freed (NIH/NCI, Rockville, MD).

pGinSin, an FIV-GFP vector was a kind gift of Dr. E. Poeschla (Mayo Clinic, Rochester, MN)[104]. pSMPU-18x21-EGFP is an eGFP reporter plasmid with a minimal HTLV promoter and 18 copies of the 21-bp HTLV tax response elements upstream of the promoter (Zhang et al., 2006). pSMPU-18x21-EGFP and pLV-Tax, encoding lentivirus tax, were kind gifts of Dr. C-Z. Giam (USUHS, Bethesda, MD)

Testing PaKiT cells for sensitivity to ZeocinTM and Geneticin. 1.5×10^5 cells of 293T cells or PaKiT cells were seeded into each well of a 12-well plate. Approximately 36 hours later, ZeocinTM (Invitrogen) or Geneticin (Invitrogen) were added at the following concentrations: 0, 10, 50, 100, 200, 300, 500, 700, 1000 $\mu\text{g/ml}$. Cell death was qualitatively examined by microscopy and photographed using an OlympusTM IX81 microscope connected to a CookeCorporationTM SensiCam^{QE}.

***P. alecto* PaKiT cell RNA isolation and cDNA synthesis.** Immortalized *P. alecto* kidney cells (PaKiT) were grown to approximately 70% confluency, washed twice with PBS, and detached with trypsin. The trypsin was quenched by the addition of DMEM with 10% CCS, and the cells were harvested by centrifugation at 1500 rpm for 10 minutes. The trypsin and media were aspirated and RNA was isolated and cDNA was

synthesized using the Superscript® III First-Strand Synthesis System (Life Technologies, Grand Island, NY) and Oligo-dT primers according to the manufacturer's recommendations. Briefly, the cells were lysed in the presence of RNaseOUT™, to inactivate ubiquitous RNA-degrading enzymes, and DNA was removed by digestion with DNaseI. cDNA was synthesized using 50 µM oligo(dT)₂₀ primers and 10 mM dNTPs by RT-PCR reaction using Superscript™ reverse transcriptase. Following RT-PCR, remaining RNA was removed by digestion with RNase H.

Primer design. Primers were designed based on the published *P. vampyrus* sequence putatively identified as an AP3B1 homolog (cite *Ensembl*). Primer APS1 is a sense primer of the sequence 5' ATGTCCAGTAACAGCTTCG 3' and starts at the initiating ATG. Primer APA3 is an antisense primer with the sequence 5' TTA CCC CTG GGA CAG GAC AGG 3' which encompasses the end of the mRNA, including the stop codon (TAA). These two primers were designed to amplify the full-length 3300 base pair mRNA encoding the *P. alecto* AP3B1 homolog.

Amplification of *P. alecto* AP3B1 from PaKiT cDNA. *P. alecto* AP3B1 was amplified from PaKiT cDNA using Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions using 4 µl of cDNA and 1 µl each primers derived from published *P. vampyrus* sequence data in *Ensembl* and from *P. alecto* transcriptome data (courtesy of L.F. Wang and M. Tachedjian, CISRO/AAHL, Australia) . The PCR reaction was carried out under the following conditions: denaturation at 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 3 minutes. The samples

were stored 4°C until the next day when an aliquot was run on a 0.5% agarose gel with 0.05% ethidium bromide to visualize a DNA band of the expected size of 3300 base pairs.

TOPO cloning. 1.5 µl of the PCR products obtained by amplification of PaKiT cDNA using the primers APS1 and APA4 were cloned into the Topo TA Cloning® kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Following a 5 minute incubation at room temperature, One Shot® Top10 chemically competent *E. coli* (Life Technologies, Grand Island, NY) were transformed with 2 µl of the TOPO TA Cloning® reaction and plated on kanamycin containing LB-agarose plates. Colonies were grown in 5 ml of L-broth overnight, harvested by centrifugation and plasmid DNA was isolated using the QIAprep spin miniprep kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The isolated plasmids were screened for the insert by sequencing using Big Dye 3.1 using the TOPO forward and reverse primers supplied by the manufacturer for the 5' and 3' segments. The obtained sequences were used to design the primers APF1 (5' GGATAGAATAGATTTGATTAC 3') (sense) and APR1 (5' GGTGGCTGAATATTAACATTGA 3') (antisense), which were used to sequence the middle regions of the AP3B1 clone.

Subcloning of the AP3B1 hinge region. The 500 base pair hinge region of AP3B1 was amplified from the full-length clone using Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA) as described above using 2 µl each of the primers HF2 (5' CGACACGCGTCCTGACCCATCAGTGCG 3') and HR2 (5' ACGACTCGAGTCTATCTTGCTTCGTTTTTTTTTTTTC 3') These primers are designed to amplify a 146 base pair fragment of the *P. alecto* AP3B1 gene corresponding to the "hinge" region of the human AP3B1 gene. Following amplification, the PCR

product was analyzed by gel electrophoresis, and, once found to contain a clean band of the appropriate size, was cloned into TOPO TA and screened by sequencing with the manufacturer supplied TOPO TA sequencing primers. A positive clone containing the insert was selected and the insert was subcloned into a pCAGGS vector containing a FLAG tag (pCAGGS-FLAG, a kind gift of W. Sun and Dr. A. P. Schmitt, Penn State University, State College, PA)

2.4 Results

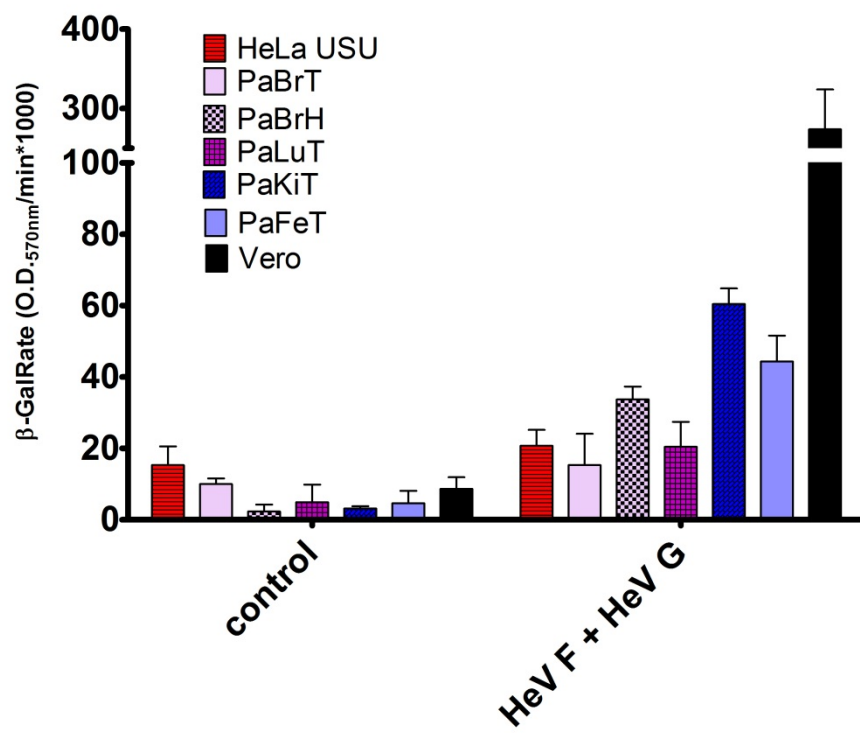
Hendra virus glycoprotein-mediated cell-cell fusion with bat-derived target cells. To determine if the well-established viral glycoprotein mediated cell-cell fusion assay could be applied to study henipavirus glycoprotein mediated cell-cell fusion with bat derived cells as targets, henipavirus receptor negative HeLa-USU cells were transfected with expression plasmids encoding the attachment (G) and fusion (F) glycoproteins of HeV, and infected with a recombinant vaccinia virus encoding the bacteriophage T7 RNA polymerase. Receptor expressing Vero target cells and bat-derived cell lines were infected with a recombinant vaccinia virus encoding a lacZ reporter gene under a T7 promoter. Receptor negative HeLa-USU target cells were included as a negative control. The cell populations were mixed and the fusion rates were measured by reporter gene activity based on colorimetric conversion of chlorophenol red- β -D-galactopyranoside (CPRG). It was found that all cell lines derived from *P. alecto* flying foxes were able to support HeV glycoprotein mediated fusion in this assay (**Fig. 1A**), with the exception of brain derived SV40 immortalized cells (PaBrT). *R. aegyptiacus* (Egyptian fruit bat) derived RO5T cells however, were not found to be fusogenic (data not shown). To

investigate why RO5T cells did not support fusion, the cells were either transfected with an expression plasmid encoding the HeV receptor human ephrin-B2 under a chicken- β -actin promoter, or infected with recombinant vaccinia vMB2 encoding human ephrin-B2 at an MOI of 10 and the assay was repeated (**Fig. 1B**). It was found that

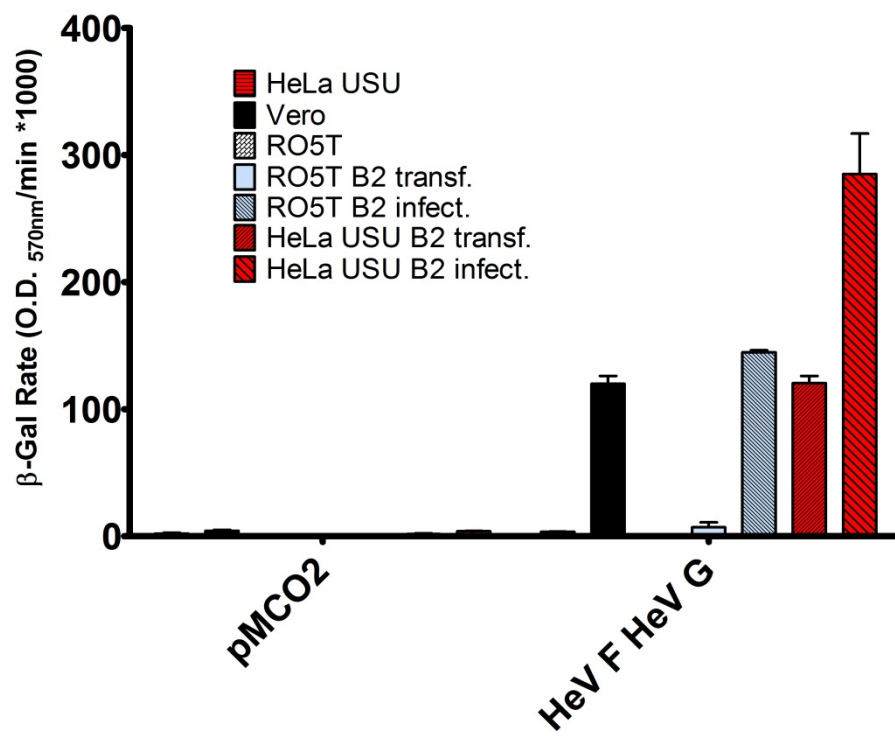
Figure 1. Hendra virus glycoprotein fusion with bat-derived target cells.

HeV F and G were co-transfected into HeLa-USU effector cells which were then infected with recombinant vaccinia virus vTF7.3 encoding T7 polymerase. Each bat-derived target cell population, as well as receptor-negative HeLa-USU cells and receptor-positive Vero cells were infected with vaccinia virus vCB21R containing lacZ under a T7 promoter and allowed to fuse with effector cells. After 2.5 hours at 37°C, the cells were lysed and β -galactosidase activity was quantitated. (A) HeV mediated fusion with *P. alecto* cell lines. (B) HeV mediated fusion with *R. aegyptiacus* cell line RO5T and HeLa-USU cells either untransfected, transfected with an ephrin-B2 plasmid, or infected with recombinant vaccinia virus vMB2 containing ephrin-B2.

A



B

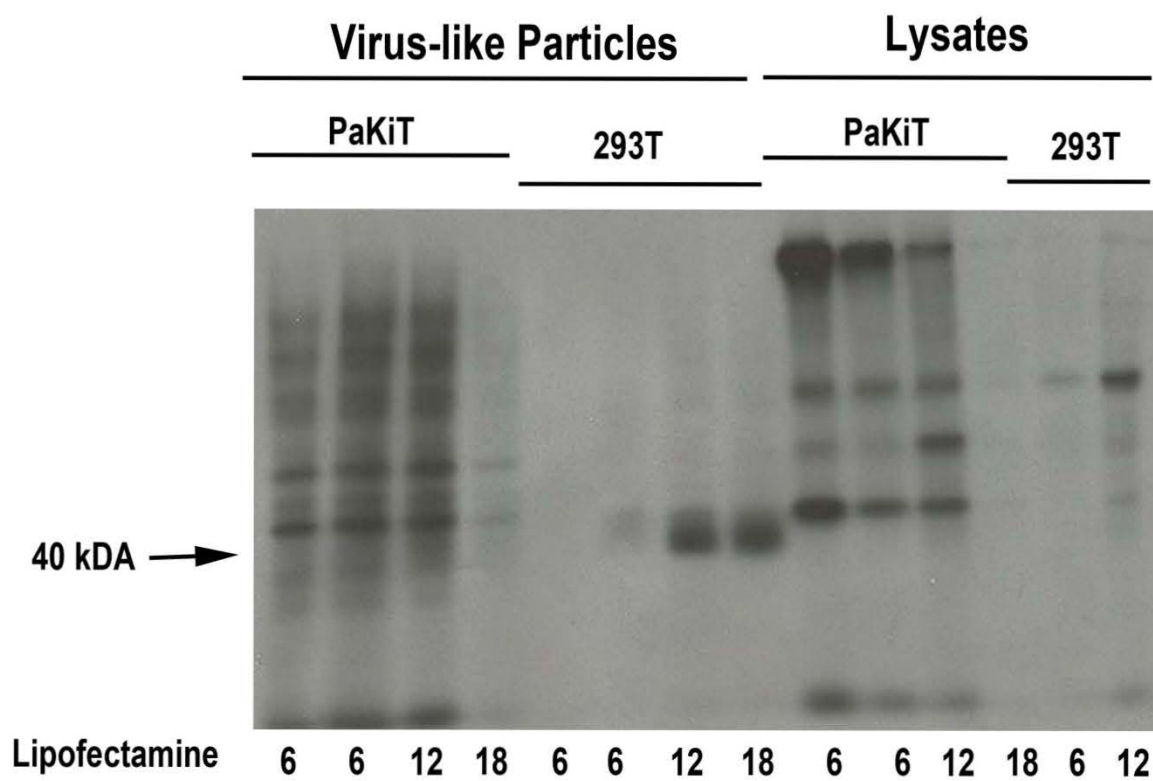


expression of the viral receptor by recombinant vaccinia virus resulted in HeV glycoprotein mediated fusion with RO5T target cells while plasmid-based expression did not, most likely due to poor transfection efficiency of RO5T cells.

Virus-like Particle (VLP) Budding assay. The VLP budding assay has been successfully used to study the assembly and budding of highly pathogenic viruses in BSL-2 facilities studying the behavior of the matrix protein in the absence of live virus [105, 106]. If it can be carried out in bat-derived cells, it may provide a safe method with which to study the dynamics of viral egress from bat cells, not only relative to the budding dynamics in human cells, but also comparing the dynamics in *R. aegyptiacus* cells to *P. alecto* cells, and, in the case of *P. alecto*, comparing bat cells derived from different organs. Increased budding efficiency could lead to increased transmission of virus and may contribute to zoonotic outbreaks. Alternatively, decreased levels of particle release from bat cells might indicate a non-immune mechanism of control of viral spread in bats mediated by bat-specific cellular factors. Initial attempts to observe NiV M-mediated VLP production by PaKiT cells were unsuccessful (data not shown), and it was determined to be due to a lack of protein production in transfected cells, presumably due to inefficient transfection. To address this possibility, a budding assay was set up to optimize the amount of LipofectamineTM which would result in highest protein production. It was found that PaKiT cells were more sensitive to high levels of LipofectamineTM than 293T cells, and that, regardless of the amount of LipofectamineTM used, no detectable expression of NiV M occurred (**Fig. 2**). This necessitated an attempt to determine more optimal conditions for PaKiT cell transfection.

Figure 2. Virus-like particle budding assay.

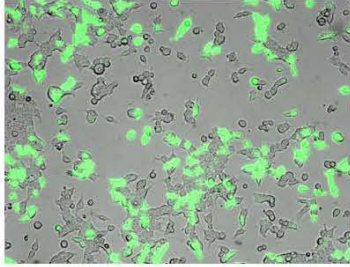
293T or PaKiT cells were transfected with 2 µg/dish of NiV M and increasing levels (6-18 µl) of LipofectamineTM. After overnight incubation the cell were labeled with ³⁵S methionine and cysteine. 18 hours after applying label, cell lysates were prepared and immunoprecipitated with S-protein agarose beads (EMD Biosciences Inc.). VLPs were harvested from the culture supernatant as detailed in the methods. Lysates and VLPs were separated by SDS-PAGE gel electrophoresis and visualized by autoradiography.



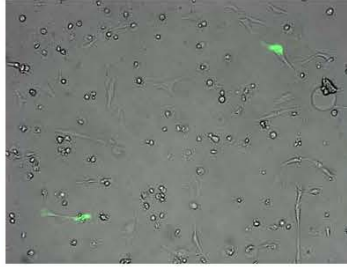
Plasmid transfections of bat-derived cell lines. To first determine if there was one bat-derived cell line which was more permissive to transfection than the others, all *P. alecto* and *R. aegyptiacus* cell lines were transfected with a reporter plasmid expressing enhanced Green Fluorescent Protein (eGFP) under the control of a chicken- β -actin promoter (D. Weir, USUHS) using LipofectamineTM and PlusTM Reagent (Invitrogen). Transfection efficiency and eGFP expression level were qualitatively evaluated over a 48 hour period by fluorescent microscopy. Transfection was successful in all cell lines, though the both the efficiency of transfection, as well as the intensity of eGFP were very low compared to 293T cells (**Fig. 3**). Based on this information, follow up experiments made use of the PaKiT cell line. To determine if other transfection platforms might be more successful, the transfections were repeated using calcium phosphate as well as (Roche Diagnostics GmbH, Mannheim, Germany), also using the eGFP reporter plasmid described above. As was seen with LipofectamineTM and PlusTM Reagent, the transfection efficiencies of bat derived cell lines with calcium phosphate and the expression of the eGFP reporter gene was low when qualitatively compared to 293T cells (**Fig. 4**). No data is shown for FugeneTM6 transfections of bat cells as all cells transfected and exposed to the FugeneTM6 reagent became unviable. Since much of the cellular biology of bats is not yet understood, it was unclear if the low levels of eGFP expression in these experiments was due purely to poor transfection efficiency, or if perhaps the phCMV promoter on the reporter plasmid is not favored by bat polymerases. To address this possibility, *P. alecto* kidney-derived PaKiT cells were transfected with three GFP-expressing reporter plasmids, each under the control of a

Figure 3. Transfection of bat-derived cell lines with eGFP reporter plasmid pCAGGS-eGFP. 293T cells, *P. alecto* and *R. aegyptiacus* derived cell lines were transfected with 2 µg of pCAGGS-eGFP reporter plasmid. 48 hours post transfection reporter gene expression was qualitatively examined by fluorescent microscopy.

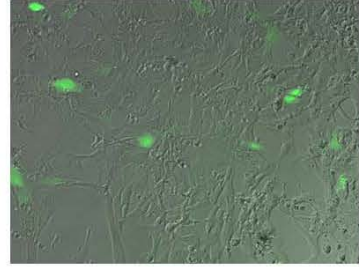
293 T



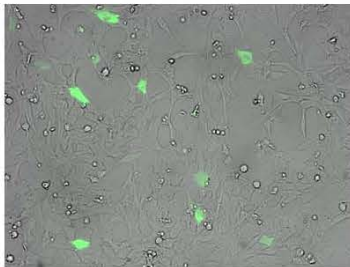
PaBrH



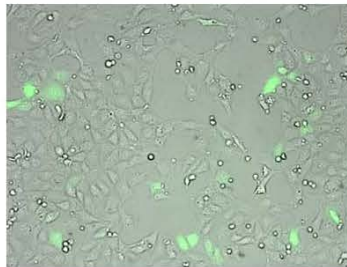
PaBrT



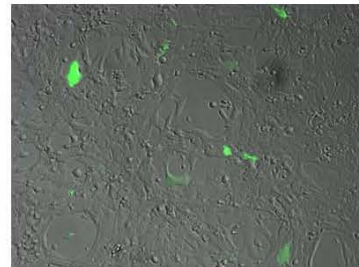
PaFeT



PaKiT



PaLuT



RO5T

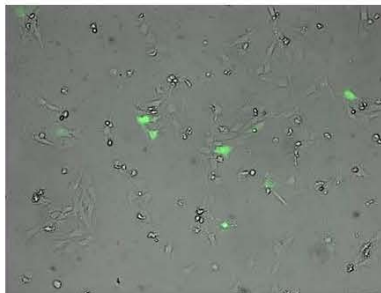
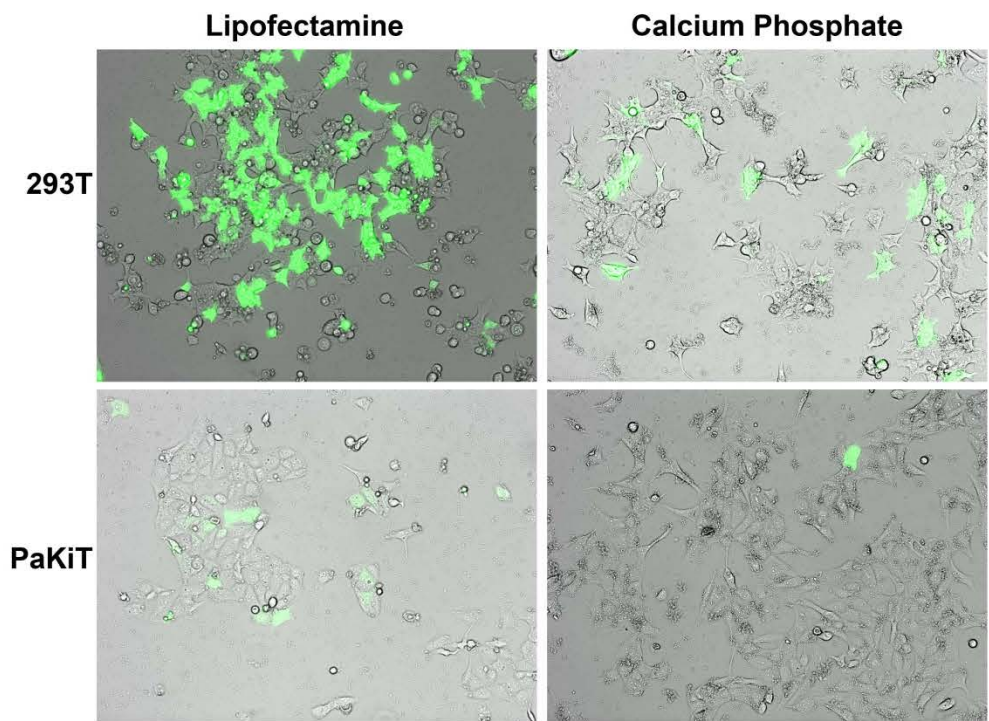


Figure 4. Calcium Phosphate transfections of PaKiT cell line.

293T cells and PaKiT cells were transfected with the reporter plasmid pCAGGS-eGFP (2 μ g) using LipofectamineTM or calcium phosphate. 48 hours after transfection relative transfection efficiency was qualitatively examined using fluorescent microscopy.



different promoter. Plasmid pGinSin contains an FIV promoter, pMxGFP contains an MMLV promoter, and pSMPU 18X21 GFP contains 18 copies of the HTLV LTR which is dependent on the co-transfection of a pBC12Tax plasmid encoding HTLV Tax for expression. While transfection efficiencies of PaKiT cells were again uniformly lower than those of 293T cells, it was encouraging that GFP expression from each plasmid was evident (**Fig. 5**), indicating that FIV, MMLV, or HTLV might serve as platforms for retroviral gene transduction of bat cells, providing an alternative to HIV-1 based systems which are restricted in these cells.

Antibiotic susceptibility of PaKiT cell line. Due to the poor plasmid transfection ability of the *P. alecto* PaKiT cell line, recombinant gene expression by plasmid transfection has not been successful. Detection of exogenous gene expression in bat cells might be facilitated by the presence of an antibiotic resistance gene on the plasmid of interest, which would allow selection of transiently or stably transfected cells. To this end, we sought to determine the susceptibility of the PaKiT cell line to two antibiotics, geneticin and ZeocinTM. ZeocinTM mediated cell death is caused by the agent intercalating into and cleaving cellular DNA. Resistance to ZeocinTM is conferred by the product of the *Streptoalloteichus hindustanus* derived *Sh ble* gene product, which binds to and prevents ZeocinTM from intercalating into DNA. Geneticin is an aminoglycoside which inhibits protein synthesis in both prokaryotic and eukaryotic cells. Geneticin resistance is mediated by the *neo^R* gene encoding an aminoglycoside phosphotransferase. By serial dilution of the antibiotics in DMEM-10 and microscopic examination, it was determined that the PaKiT cell line is sensitive to ZeocinTM at 50 µg/ml as well as to geneticin at 200 µg/ml (**Fig. 6**). This will facilitate the selection of PaKiT cells in a culture which have

been successfully transfected with a plasmid containing a gene of interest as well as a ZeocinTM or geneticin resistance marker, and can also be used to establish a clonal PaKiT-derived cell line stably transfected with the plasmid.

Figure 5. Transfection of PaKiT cell line with lentivirus-derived eGFP reporter plasmids. 293T cells and PaKiT cells were transfected with three EGFP reporter plasmids utilizing three different lentivirus promoters. Reporter gene expression at 48 hours post infection was qualitatively examined by fluorescent microscopy.

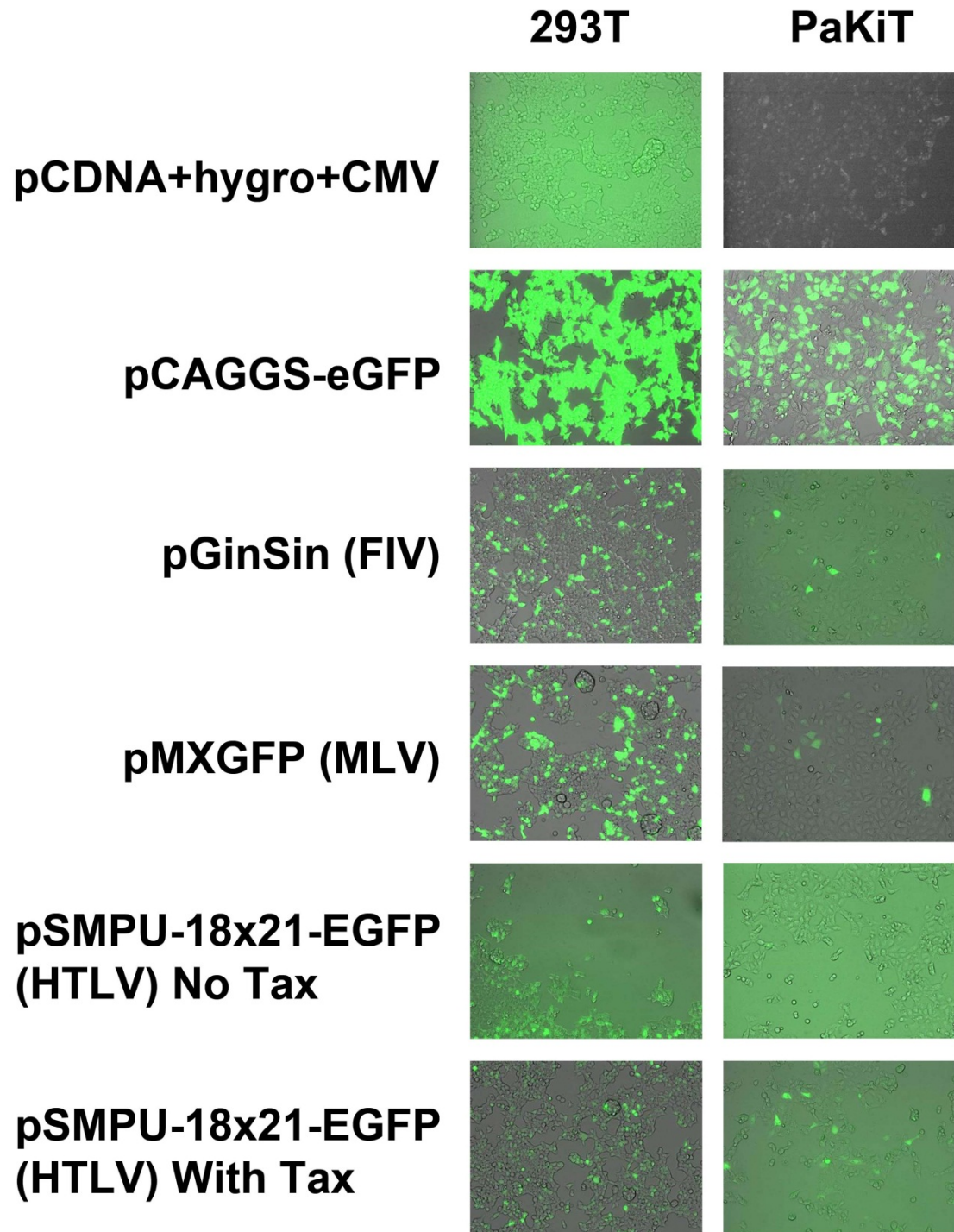
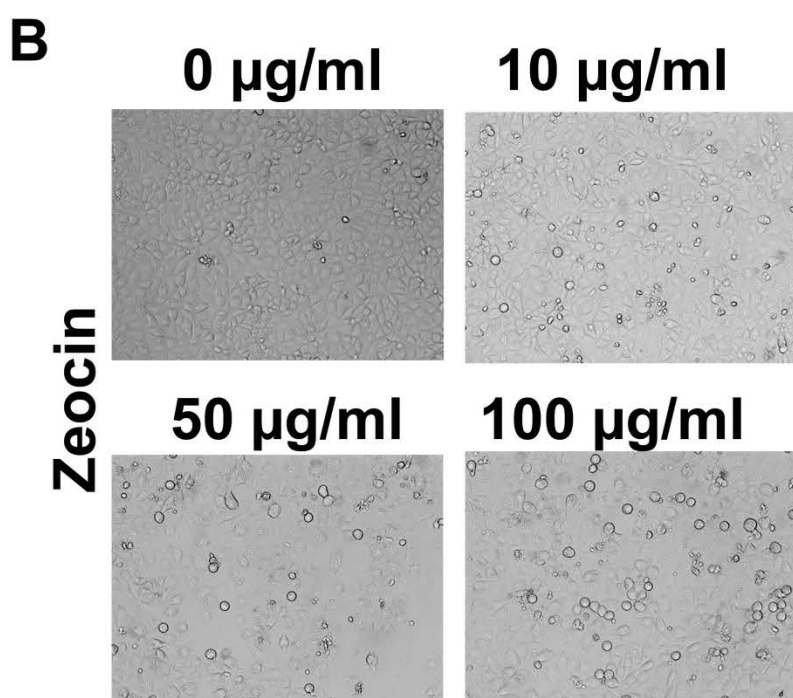
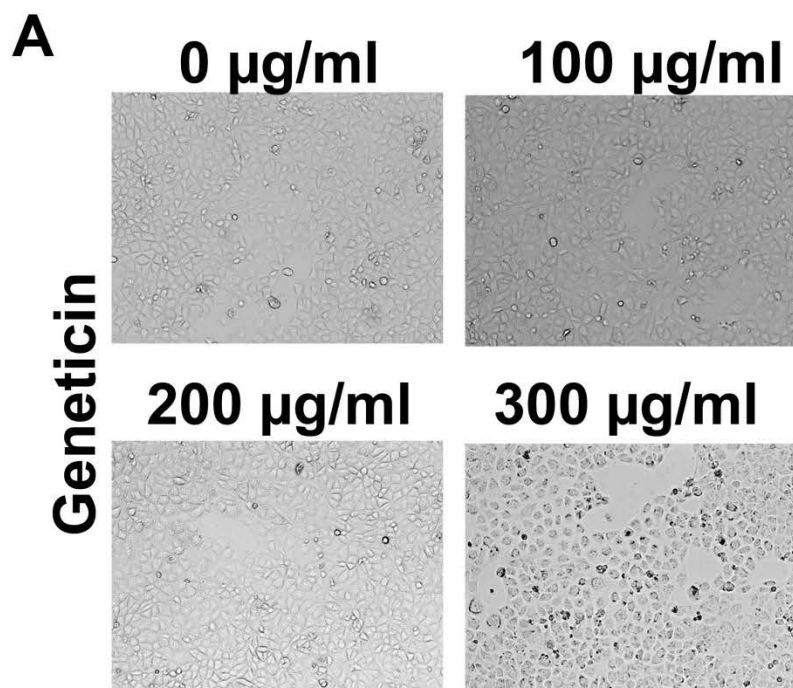


Figure 6. Susceptibility of PaKiT cell line to Zeocin and Geneticin.

293T cells and PaKiT cells in 6-well plates were treated with either no antibiotic or increasing amounts of Geneticin (A) or ZeocinTM (B). Loss of cell viability was qualitatively examined by microscopy.



Cloning of the *P. alecto* homolog of human AP3B1. Human AP3B1, a subunit of an intracellular adaptor protein complex, has been shown to interact with NiV M in a manner that prevents it from mediating virus-like particle assembly and egress (W. Sun and A.P. Schmitt, manuscript in preparation). The protein consists of three domains, a “head” domain, a “hinge” domain, and an “ear” domain. The specific interaction of AP3B1 with the NiV M protein was mapped to the “hinge” region of the protein, specifically two stretches of amino acids in the “hinge”, from amino acids 643-705 and amino acids 753-809, termed hinge 1 and hinge 3. The expression of only these fragments of the AP3B1 protein results in a strong binding to NiV M and abrogation of VLP budding. This region is rich in charged amino acids, particularly serine residues, which Sun and colleagues have postulated to be critical for the interaction with NiV M. It was thus of great interest to us to determine if the *P. alecto* homolog of this cellular protein might demonstrate an even greater affinity for NiV M and might be more effective in abrogating NiV budding, thereby perhaps playing a role in the control of NiV infections in bats, and possibly representing a platform for the design of small molecule inhibitors of NiV budding. To clone and sequence the bat homolog of AP3B1, RNA was isolated from the *P. alecto* kidney cell line PaKiT and cDNA was synthesized using oligo dT primers. The open reading frame of AP3B1 was amplified using primers designed based on available *P. vampyrus* mRNA sequences published in *Ensembl* and based on *P. alecto* transcriptome data from Dr. L-F Wang (CSIRO, AAHL). The resulting PCR product was cloned into a TOPO TA vector and sequenced. While this gene is well conserved across species, the sequence data indicates that the *P. alecto* protein may differ slightly but significantly from the human protein in the “hinge” domain that is thought to

mediate interactions with the henipavirus M protein. As illustrated in **Figure 7**, in the *P. alecto* sequence there are five amino acid differences in the hinge 1 region, 17 amino acid differences in the hinge 2 region, and 10 amino acid differences in the hinge 3 region when compared to its human counterpart. It is noteworthy that the *P. alecto* AP3B1 is overall very similar to human AP3B1, sharing a 93% sequence identity, and that the greatest level of sequence divergence is found in the “hinge” region, which is predicted to interact with NiV M. The functional consequences of these amino acid differences remain to be determined.

Figure 7. Alignment of the *P. alecto* AP3B1 homolog with human AP3B1.

The sequence of the cloned *P. alecto* AP3B1 homolog was aligned with the sequence of human AP3B1 using PRALINE (<http://www.ibi.vu.nl/programs/pralinewww/>).

Conserved amino acids are highlighted in red, nonconserved amino acids are highlighted in orange, green, or blue, in increasing order of divergence.

Unconserved 1 2 3 4 5 6 7 8 9 10 Conserved

	10	20	30	40	50
P_allecto_AP3B1	MSSNSFAYNE	QSGGGEATEL	GQEATAITISF	SGAFGLFSSD	LKKNEDLKQM
_H_sapiens_AP3	MSSNSFPYNE	QSGGGEATEL	GQEATSTISF	SGAFGLFSSD	LKKNEDLKQM
Consistency	*****3***	*****	*****6***	*****	*****
	60	70	80	90	100
P_allecto_AP3B1	LESNKDSAKL	EAMKRIVGMI	AKGKNASELF	PAVVKNVASK	NIEIKKLVYV
_H_sapiens_AP3	LESNKDSAKL	DAMKRIVGMI	AKGKNASELF	PAVVKNVASK	NIEIKKLVYV
Consistency	*****6*	*****	*****	*****	*****
	110	120	130	140	150
P_allecto_AP3B1	VLVRYAEQQ	DLALLSISTF	QRALKDPNQL	IRASALRVLS	SIRVPIIVPI
_H_sapiens_AP3	VLVRYAEQQ	DLALLSISTF	QRALKDPNQL	IRASALRVLS	SIRVPIIVPI
Consistency	*****	*****	*****	*****	*****
	160	170	180	190	200
P_allecto_AP3B1	MMLAIKEASA	DLSPYVRKNA	AHAIQKLYSL	DPEQKEMLE	VIEKLLKDKS
_H_sapiens_AP3	MMLAIKEASA	DLSPYVRKNA	AHAIQKLYSL	DPEQKEMLE	VIEKLLKDKS
Consistency	*****	*****	*****	*****	*****
	210	220	230	240	250
P_allecto_AP3B1	FLVAGSVVMA	FEEVCPDRID	LIHKNYRKLC	NLLVDVEEWG	QVVIHMLTR
_H_sapiens_AP3	FLVAGSVVMA	FEEVCPDRID	LIHKNYRKLC	NLLVDVEEWG	QVVIHMLTR
Consistency	*****	*****	*****	*****	*****
	260	270	280	290	300
P_allecto_AP3B1	YARTQFVSPW	KEDDGLQDNE	KNFYESDDEQ	KEKTE- KKKF	YTMDDPHRL
_H_sapiens_AP3	YARTQFVSPW	KEDDGLQDNE	KNFYESDDEQ	KEKTD KKKF	YTMDDPHRL
Consistency	*****	***3*2*6*2	*****6*	***60***0	*****
	310	320	330	340	350
P_allecto_AP3B1	TRNTKPLLQS	RNAAVVMABA	QLYWHIAPKS	EAGIISKSLV	RLLRSNREVQ
_H_sapiens_AP3	TRNTKPLLQS	RNAAVVMABA	QLYWHISPKS	EAGIISKSLV	RLLRSNREVQ
Consistency	*****	*****	*****6*	*****	*****
	360	370	380	390	400
P_allecto_AP3B1	YIVLQNIATM	SIQRKGMFEP	YLSFYVRST	DPTMIKTLKL	EILTNLANE
_H_sapiens_AP3	YIVLQNIATM	SIQRKGMFEP	YLSFYVRST	DPTMIKTLKL	EILTNLANE
Consistency	*****	*****	*****	*****	*****
	410	420	430	440	450
P_allecto_AP3B1	NISTLLREFQ	TYVKSQDKQF	AAATIQTIGR	CATNLS EVTD	TCLNGLVCLL
_H_sapiens_AP3	NISTLLREFQ	TYVKSQDKQF	AAATIQTIGR	CATNLS EVTD	TCLNGLVCLL
Consistency	*****	*****	*****	***32***	*****
	460	470	480	490	500
P_allecto_AP3B1	SNRDEIVVAE	SVVVIKKLLQ	MQPAQHGEII	KHMAKLLDSI	TVPVARASIL
_H_sapiens_AP3	SNRDEIVVAE	SVVVIKKLLQ	MQPAQHGEII	KHMAKLLDSI	TVPVARASIL
Consistency	*****	*****	*****	*****	*****
	510	520	530	540	550
P_allecto_AP3B1	WLTGENCERV	PKIAPDVLRK	MAK NFTS EDD	LVKLQILNLG	AKLYLTNSKQ
_H_sapiens_AP3	WLTGENCERV	PKIAPDVLRK	MAK SFTS EDD	LVKLQILNLG	AKLYLTNSKQ
Consistency	***3*****	*****	***5*****	*****	*****

	560	570	580	590	600
P_alecto_AP3B1		TKLLTQYILN	LGKRDQNYDI	RDRTRFIRQL	IVPNEKSGAL	SKYAKKIFLA				
_H_sapiens_AP3		TKLLTQYILN	LGKRDQNYDI	RDRTRFIRQL	IVPNEKSGAL	SKYAKKIFLA				
Consistency		*****	*****	*****	*****	*****				
	610	620	630	640	650
P_alecto_AP3B1		QKPAPLLESP	YKDRDHFQLG	TLSTLTNTKA	TGYLELSNWP	EVAPDPSVRN				
_H_sapiens_AP3		QKPAPLLESP	FKDRDHFQLG	TLSTLTNTKA	TGYLELSNWP	EVAPDPSVRN				
Consistency		*****	6*****	*****3**	*****	*****				
	660	670	680	690	700
P_alecto_AP3B1		VEVIELAKEW	TPAGKAKKEN	PAKKFYSESE	EEEDSSDSSS	DSESESGSES				
_H_sapiens_AP3		VEVIELAKEW	TPAGKAKQEN	SAKKFYSESE	EEEDSSDSSS	DSESESGSES				
Consistency		*****	*****5**	3*****	*****	*****4**				
	710	720	730	740	750
P_alecto_AP3B1		EEQDEEGDSS	EESEEDSSSE	EESESGSESE	V-----ENRR	DKNSKTKGKG				
_H_sapiens_AP3		EEQDEEGEG	DSNEDSSSDS	EEQDSESGR	ESGLENKRTA	KNSKAKGKS				
Consistency		**3**4*644	*45464*444	*446444444	2000005433	36***4***4				
	760	770	780	790	800
P_alecto_AP3B1		DSEYGEKENE	NSETSDSSNE	ESSSIESSSS	DSESESESEN	EESRKRVTKE				
_H_sapiens_AP3		DSEDGEKENE	KSKTSDSSND	ESSSIEDSSS	DSESESESES	EESRRVTKE				
Consistency		***0*****	4*5*****6	*****6**	*****3*5	*2***6***				
	810	820	830	840	850
P_alecto_AP3B1		KEKKKTQDR	KFVTKDVSLL	DLDDFNPVST	PVALPMPAFS	PSLIADLEGL				
_H_sapiens_AP3		KEKKKTQDR	TFLTKDVSLL	DLDDFNPVST	PVALPTFALS	PSLMADLEGL				
Consistency		***30****	3*6*****	*****	***3*4*	***5*****				
	860	870	880	890	900
P_alecto_AP3B1		NLSTSSSVIS	VSTFVFVPMK	THVLLHRMSG	KGLAANYFFP	RQPCIFGDKM				
_H_sapiens_AP3		NLSTSSSVIS	VSTPAFVPTK	THVLLHRMSG	KGLAANYFFP	RQPCIFGDKM				
Consistency		4*****	***5***3*	*****	*****	*****				
	910	920	930	940	950
P_alecto_AP3B1		VSVQITLSNT	TDRKIENIHT	GQKKLTGMQ	MHVFNPIESL	EPEGSITVSM				
_H_sapiens_AP3		VSIQITLNNT	TDRKIENIHI	GEKKLIDGMK	MHVFNPIDSL	EPEGSITVSM				
Consistency		**8***5**	*****3	*6***3*5	*****6**	*****				
	960	970	980	990	1000
P_alecto_AP3B1		SIDFC DSTQT	ASFQLCTKDD	CFNVNIQPPV	GELLLPV AIS	EKDFKKEQGM				
_H_sapiens_AP3		SIDFC DSTQT	ASFQLCTKDD	CFNVNIQPPV	GELLLPVAMS	EKDFKKEQGV				
Consistency		*****	*****	*****	*****5*	*****5				
	1010	1020	1030	1040	1050
P_alecto_AP3B1		LTGMNETSTI	IIVAPQNFTS	SVILQKVNVV	ANVGVPVPSGQ	DNIHRFAAKT				
_H_sapiens_AP3		LTGMNETSAV	IIAAPQNFTP	SVIFQKVNVV	ANVGAVPSGQ	DNIHRFAAKT				
Consistency		*****48	**5*****3	***4*****	***5*****	*****				
	1060	1070	1080	1090	
P_alecto_AP3B1		VHSGSLMLVT	VELKEGSTAQ	LIINTEKTVI	GSVLLRELKP	VLSQG				
_H_sapiens_AP3		VHSGSLMLVT	VELKEGSTAQ	LIINTEKTVI	GSVLLRELKP	VLSQG				
Consistency		*****	*****	*****	*****	*****				

2.5 Discussion. Since bats are known to carry a wide variety of viruses of diverse Orders, and of particular relevance to us, are known to be the reservoir host species of the henipaviruses, it is of great interest to determine the dynamics of viral infection in bat cells. With the recent establishment of the first *R. aegyptiacus* and *P. alecto* immortalized cell lines there are now tools to address this area of research. First, it was of interest to evaluate the cell lines in the context of a HeV glycoprotein mediated cell-cell fusion assay. It was found that all bat-derived cell lines with the exception of the *R. aegyptiacus* cell line RO5T were able to support fusion and therefore can be used to study henipavirus fusion with cells of its natural reservoir host safely in a BSL-2 setting. The inability of the RO5T cell line to mediate fusion is interesting, and the cause of this is not yet known. Since exogenous expression of the henipavirus receptor ephrin-B2 by recombinant vaccinia virus infection allowed RO5T to mediate fusion, it is possible that this cell line lacks the required viral receptors. This will be examined by attempting to detect ephrin-B2 and -B3 expression by western blot or radio-immunoprecipitation.

Attempts to study the budding of NiV in *P. alecto* kidney cells (PaKiT) however, were unsuccessful due to an inability to produce recombinant NiV M in PaKiT cells through plasmid transfection. Further investigations demonstrated that PaKiT cells, and in fact all five *P. alecto* cell lines and the *R. aegyptiacus* cell line RO5T were refractory to transfection using LipofectamineTM and PlusTM Reagent (Invitrogen), FugeneTM 6 (Roche Diagnostics GmbH), as well as calcium phosphate. Several options remain open. Exogenous gene expression using a retroviral vector is a possibility, and this work has shown that the promoter systems of HTLV, FIV, and MMLV are all functional in PaKiT

cells, indicating that transduction by a retroviral vector may be successful. A second possibility is to enrich the population of cells that were successfully transfected by introducing an antibiotic resistance gene along with the gene of interest on a plasmid. This work presents two options for this approach as it has established that PaKiT cells are susceptible to killing by both ZeocinTM and Geneticin at 50 µg/ml and 200 µg/ml, respectively.

Future work to optimize the expression of genes of interest in bat cells will require a multi-pronged approach. Other transfection reagents such as the non-liposomal transfection reagent Effectene (Qiagen), or the novel magnet-based transfection system MagnetofectionTM (Origene) could be attempted, and electroporation can also be considered. Stably transfected PaKiT cell line derivatives can be established using the Zeocin or Geneticin resistance genes, or the antibiotics could be used to select for transiently transfected populations to enhance the detection of protein expression. Once detectable exogenous gene expression in bat cell lines is achieved, much work can be done to piece together the puzzle of bat cell biology and its influence on viral dynamics. One priority is to examine the cellular trafficking of matrix protein in bat cells as compared to human cells. It is known that deletion of late domain sequences in NiV M result in an abrogation of budding and a nuclear retention phenotype [27, 106, 107]. Examining the effect of late domain deletion in NiV M in bat cells would reveal if matrix trafficking and budding in cells of the reservoir host are analogous to or distinct from these processes in human cells. Additionally, it has been suggested that the rather complex nature of maturation of the F glycoprotein by endosomal recycling may be due to a differential expression pattern of bat cellular proteases, or the presence of alternate

proteases in bat cells capable of cleaving the henipavirus F glycoprotein [49]. This possibility can be addressed by examining F glycoprotein cleavage in bat cells by first determining if an endosomal recycling step is required or not. This work would shed light not only on the dynamics of viral replication in bat cells, but also on the basic cell biology of bats, as viruses have long been used to elucidate the details of complex cellular processes.

A full-length AP3B1 homologous open reading frame was successfully amplified from *P. alecto* PaKiT cell line cDNA. It was found to share an overall amino acid sequence identity of 93% with human AP3B1, a high level of similarity which was expected due to the generally highly conserved nature of AP3B1 across various animal species. However, in the “hinge” region of the protein, the area known to mediate the interaction of AP3B1 with NiV M (W. Sun and A.P. Schmitt, manuscript in preparation), there are several relevant amino acid changes. Notably, there are several additional serine residues in this region, particularly in the “hinge 2” subdomain, a region which, in the human homolog, does not exhibit significant NiV M binding or inhibition of NiV M budding.

Future experiments to determine if these amino acid differences modify the interaction of this protein with viral matrix proteins, Flag-tagged polypeptides corresponding to the hinge 1, 2, and 3 regions of *P. alecto* AP3B1 will be expressed in 293T cells and used to co-immunoprecipitate NiV M. The ability of the bat sequence derived polypeptides to bind and co-IP NiV M will be compared to that of human sequence derived polypeptides. To determine what effects, if any, these differences have on assembly and budding, NiV M budding assays will be carried out in the presence of these overexpressed polypeptides and the relative budding efficiency will be compared to

293 T cells in the presence of the human polypeptides and empty expression vector as a control. If it is found that the bat-derived sequence exhibits greater affinity for NiV M, and a more efficient inhibition of budding than the human sequence, then this could represent one aspect of the complex control of virus infection in bats. Additionally, it could serve as a platform for the design of small molecule inhibitors of henipavirus budding which could be developed as an antiviral agent.

Implications. Bats have long been the misunderstood objects of irrational fears and superstition, and surely the recent revelation of bats as carriers of a vast amount of deadly viruses and other agents which threaten human health, will add another dimension to the fear of bats. However, it is true that people fear that which they do not understand, and the only way to counteract the negative image of bats is to emphasize the positive and irreplaceable ecological roles of bats. Bats are critical components of the ecosystem of our planet and it is important not to take the discovery of their role in the ecology of virus infections as incentive to hunt them, or fail to fight for their preservation. Fruit bats consume seeds in process of foraging and, due to large flight ranges, scatter these seeds over a wide range of geographical area, encouraging the growth and spread of forests. On small Pacific islands that have a relative paucity of vertebrate species, flying foxes can be the only available animals that possess the body size required to eat and disperse the seeds of large fruits [108]. Fruit eating bats are also important for the re-growth of forest lost due to fires or climactic events as they eat and distribute the seeds of so called “pioneer species” of plants which serve as the foundation for new growth (reviewed in [109]), for example, the kapok tree on the pacific island of Samoa is entirely dependent on *P. tonganus* for pollination, while kapok trees in Australia do not have this restriction

([110]. Additionally, nectar and pollen are carried by bats that are the main mediators of the pollination of nocturnally blooming plants, and indeed, some plants have evolved specific flower shapes, positions, and odors to attract bats, and bats are known to pollinate at least 360 species of plants[111]. *P. rufus*, a large fruit bat, plays a key role in maintaining the forests of Madagascar, and is likely the main pollinator of the unique, ancient and critically endangered baobab tree. As in other regions where the continuity of the forest has been disrupted due to climactic events such as hurricanes, or human activities such as agriculture, the long flight ranges of bats make them crucial for maintaining plant populations, which might otherwise die out due to a lack of nearby pollination partners. Other important bat pollinated plants include wild bananas, *Agave tequilana* a cactus of great economic and cultural significance in Mexico, as well as *Eucalyptus* trees in Australia, and Mangrove trees in south-east Asia, which are the foundation of a mangrove community habitat of great species richness (reviewed in [111]). The loss of nectar and fruit eating bats would have a snowball effect, causing in the loss of a number of economically and ecologically important plants and the local habitats they form.

Insectivorous bats are also vitally important to maintenance of the ecosystem. A study conducted in a coffee agroforest in Mexico found that, by restricting the access of bats to coffee plants by applying netting, the arthropod density on the foliage of these plants in the rainy season increased by 84% in an eight week period, 30% more than if the access of birds was restricted [112]. An additional study restricting the access of bats to the foliage of a Panamanian lowland tropical forest demonstrated a 153% increase in arthropod density, and a 209% increase in foliage being eaten by those arthropods, again

greatly exceeding the effect of the removal of bird predation of arthropods[113]. These studies clearly and quantifiably demonstrate the importance of bats in controlling arthropod populations, and it is clear that if bat populations were to be lost, the amount of damage done by arthropods to crops and forests would be inestimable.

Bats have a history of being misunderstood, and often are feared due to their unique appearance and nocturnal lifestyles, and increasingly, due to a reputation for being hazardous to human health. However, it must be recognized that the main factors contributing to the spillover of bat carried viruses into human or domestic animal populations are due to human activities such as deforestation, habitat encroachment, hunting, and spelunking, to name a few. Loss of bat populations would cause enormous economic and ecological losses from increased arthropod predation, decreased pollination, and decreased seed dispersal of plants, concomitant with an increased risk of arthropod-borne diseases of both animals and humans.

Bats are key members of our ecosystems and their highly threatened populations are in need of protection. An additional benefit of the recent resurgence of interest in bats as virus reservoirs is that an increased understanding of bat physiology and the details of the virus-bat host relationships on both an organismal and cell biological level may aid education and conservation efforts as well potentially offer new insights in how to control or combat virus infection in otherwise susceptible mammalian species

Chapter 3

Functional Expression and Ephrin Receptor Tropism of Cedar Virus, a Newly Discovered *Henipavirus*.

3.1 Introduction. Recently, Marsh and colleagues [114] described the isolation of a novel paramyxovirus named Cedar virus (CedPV) from pooled bat urine collected in Queensland, Australia. CedPV was found to be similar enough to HeV and NiV that it is considered a member of the *Henipaviruses*, but distinct enough from both that it is the first new species in the genus. It has also been found to be antigenically similar enough to HeV and NiV that sera against the CedPV N protein can detect HeV infection in Vero cells, and conversely, that rabbit anti-*Henipavirus* sera could detect CedPV infection in Vero cells. However, cross-neutralization of CedPV with *Henipavirus* sera was not observed. This supports the idea that there are other *Henipaviruses* circulating in animal populations as demonstrated by the identification of sera of Pteropid and non-*Pteropus* bats in China, Vietnam, and pigs in Ghana [79, 90, 115], which recognize, but do not neutralize *Henipavirus*.

One significant difference between Cedar virus and HeV and NiV lies in the phosphoprotein gene (P). In HeV and NiV, as well as some other members of the *Paramyxovirinae*, (reviewed in [116]), editing of the P gene mRNA can lead to the production of two additional proteins through the addition of nontemplated guanine residues. The products of these two edited genes are the V and W proteins, which make

essential contributions to pathogenicity by antagonizing the host cell's innate immune response. The Cedar virus P gene lacks the V open reading frame, the conserved RNA editing site as well as any V-related transcripts. This may explain the failure of CedPV to cause disease in the experimental infection of mice, ferrets, or guinea pigs.

Cedar virus was shown incapable of infecting the ephrin-B2 and -B3 negative HeLa-USU cell line [114]. However, when HeLa-USU cells stably expressing ephrin-B2 or ephrin-B3, the known HeV and NiV entry receptors, it was demonstrated that CedPV infection was permissive with ephrin-B2 expressing but not ephrin-B3 expressing target cells (REFF) This finding further demonstrated the relatedness of CedPV to the *Henipavirus* genus, yet perhaps somewhat surprising given the relatively low level of amino acid homology of the CedPV G glycoprotein in comparison to the G glycoproteins of HeV and NiV (**Table 1**). In light of these initial observations, a greater detailed examination of the CedPV glycoprotein mediated fusion activities, receptor usage and target cell tropism was now an important area to explore.

3.2 Aims and Hypotheses.

The overall goal of this work was to determine the functional properties of the CedPV F and G glycoproteins.

Specific Aim 1: To functionally express recombinant CedPV F and G glycoproteins

Hypothesis: The F and G glycoproteins of CedPV can be functionally expressed in eukaryotic cells in the absence of other viral proteins through a plasmid-based transfection of the recombinant open reading frames.

Hypothesis 2: The functionality of the F and G glycoproteins of CedPV can be quantitated using a viral glycoprotein-mediated a cell-cell fusion assay, and, like HeV and NiV, CedPV fusion will be pH independent and require the presence of both the F and G viral glycoproteins.

Hypothesis 3: CedPV G glycoprotein will be able to mediate viral glycoprotein-mediated cell-cell fusion utilizing the henipavirus entry receptor ephrin-B2, and additionally may exhibit binding to an expanded range of ephrin molecules, which will also trigger fusion.

Table 1. Amino acid percent homology comparisons of the CedPV F and G glycoproteins in comparison to HeV and NiV

	HeV F	NiV F
CedPV F	42%	43%
	HeV G	NiV G
CedPV G	29%	30%

3.3 Materials and Methods

Cell lines. HeLa-ATCC cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HeLa-USU cells have been described previously [117]. Human 293T cells were provided by Dr. G. Quinnan (Uniformed Services University). HeLa-USU-B2 and HeLa-USU-B3 which stably express only ephrin-B2 or -B3 respectively were prepared by plasmid transfection, selection and limiting dilution cloning. All cells were maintained in standard culture conditions at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) with 10% cosmic calf serum (CCS) (Hyclone, Logan, UT), 1000 units/ml penicillin/streptomycin and 2 mM L-glutamine (Quality Biological Inc., Gaithersburg, MD).

Virus-like particle budding assay. The virus-like particle assay was carried out as described in the methods of chapter 2. Briefly, 293T cells were seeded in 10cm dishes after coating with poly-D-lysine (MP Biomedicals LLC) and transfected with S-peptide tagged CedPV M, ΔL1 CedPV M, ΔL2 CedPV M, or NiV M. After overnight incubation the cells were radiolabeled with ³⁵S-methionine and cysteine, and after 18 hours supernatants were harvested and VLPs were isolated through ultracentrifugation through a sucrose cushion, floatation through a discontinuous sucrose gradient, and a pelleting step. The VLP pellet was resuspended in SDS-PAGE sample buffer and run on an SDS-PAGE gel along with the corresponding cell lysates which had been precipitated using S-protein agarose beads (EMD Biosciences Inc.) or anti-NiV M polyclonal rabbit sera (A.P. Schmitt, Penn State University). Once complete, the gel was dried and placed on imaging film (Kodak) for autoradiography.

Site-directed mutagenesis. All mutagenesis reactions were carried out using the QuickChange® II Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions and using the following PCR conditions; a melting step of 95°C for 5 minutes, followed by 11 cycles of 95°C for 30 seconds, 56°C for 1 minute and 30 seconds, then extension at 68°C for one minute per kilobase. Samples were kept at 4°C overnight and treated with the methylated-sequence specific restriction enzyme DpnI to remove parental DNA. The digested plasmids were transformed into chemically competent XL-1-blue *E.coli* cells and plated onto ampicillin containing LB-agarose plates. Colonies were grown in L-broth, and plasmid DNA was purified using the QIAprep spin miniprep kit (Qiagen, Valencia, CA) as described above. The plasmids were screened for the desired mutations by sequencing with appropriate sequencing primers. Positive clones were subcloned into pCAGGS at the SmaI restriction enzyme site.

The putative late domains of CedPV M were deleted using the following primer pairs: to delete late domain 1 and create Δ L1 CedPV M: (₇₁YQYM₇₅): CL1S (5' GGAAAGAATGAGCGAAAAACCTCTGGCTATCTGCTATGGGTTTATTGAAGAC G 3') and CL1A (5'CGTCTTCAATAAACCCATAGCAGATAGCCAGAGGTTTTTCGCTCATTCTTT CC 3'); to delete late domain 2 and create Δ L2 CedPV M: (₁₀₁FPLGVG₁₀₆): CL2S: (5' GGAAATATCCGGACCACAGCTTCAAAGACCTACAGCTCCCCCGAGG 3') and CL2A (5' CCTCGGGGGAGCTGTAGGTCTTTGAAGCTGTGGTCCGGATATTTCC 3').

Single and double amino acid substitutions were made in the putative late domains of CedPV M using the following primer pairs: for Ced PV M $_{71}YQYM_{75} \rightarrow _{71}YMYL_{75}$: CN1F (5' CGAAAAACCTCTGGCTACATGTATCTGATCTGCTATGGGTTTATTG 3') and CN1R (5' CAATAAACCCATAGCAGATCAGATACATGTAGCCAGAGGTTTTTCG 3'); for Ced PV M $_{71}YQYM_{75} \rightarrow _{71}YMYM_{75}$: CN2F (5' CGAAAAACCTCTGGCTACATGTATATGATCTGCTATGGGTTTATTG 3') and CN2R (5' CAATAAACCCATAGCAGATCATATACATGTAGCCAGAGGTTTTTCG 3'); for CedPV M $_{101}FPLGVG_{106} \rightarrow _{101}YPLGVG_{106}$: CN4F (5' GGAAATATCCGGACCACAGCTTCATACCCACTGGGCGTCGGGAAGACC 3') and CN4R (5' GGTCTTCCCGACGCCAGTGGGTATGAAGCTGTGGTCCGGATATTTCC 3').

Gene synthesis and cloning. The open reading frames of NiV matrix and CedPV matrix were submitted to Genscript™, codon optimized, and synthesized with an s-peptide tag for detection. They were subcloned into the eukaryotic expression vector pCAGGS at the EcoRV site. The open reading frames encoding the attachment (G) and fusion (F) glycoproteins of CedPV were codon optimized and synthesized by Genscript™, and were subsequently cloned into the hCMV promoter driven expression vector pcDNA+hygro+CMV [118] using the restriction enzyme site ApaI and XhoI. An s-peptide was added to the F and G open reading frames using the QuickChange site directed mutagenesis kit as described above. The primers used to add the s-peptide tag were: for CedPV F: forward primer (FstgF:

5'CAGATCTCGAGGCCACCATGAAGGAGACCGCCGCCGCCAAGTTTGAAAGGC
AGCACATGGACTCAAGCAACAAGCGGACCACTGTCC 3') and reverse primer
FstgR: (5'
GGACAGTGGTCCGCTTGTTGCTTGAGTCCATGTGCTGCCTTTCAAACCTGGCG
GCGGCGGTCTCCTTCATGGTGGCCTCGAGATCTG 3'). For CedPV G: forward
primer GstgF: (5'
ACAGATCTCGAGGCCACCATGAAGGAGACCGCCGCCGCCAAGTTTGAAAGG
CAGCACATGGACTCACTGAGCCAGCTGCAGAAAACTACC 3'), and reverse
primer (GstgR: 5'
GGTAGTTTTTCTGCAGCTGGCTCAGTGAGTCCATGTGCTGCCTTTCAAACCTG
GCGGCGGCGGTCTCCTTCATGGTGGCCTCGAGATCTGT 3'). The open reading
frames encoding the fusion glycoproteins of HeV and NiV were codon optimized and
synthesized by Genscript™ (Piscataway, NJ), with an s-peptide (N-
KETAAAKFERQHMDs-C) tag for detection. They were subsequently cloned into the
expression vector pcDNA+hygro+CMV[119]. The attachment glycoprotein (G) genes of
HeV and NiV in the expression vector pCAGGS have been previously described [120].
The genes encoding human ephrins-A1, -A2, -A3, -A4, -A5, and -B1 in the expression
vector pCMV6-XL4 were purchased from Origene™ (Rockville, MD). Amino acid
substitutions in ephrin-A2 and -A3 were introduced by site directed mutagenesis as
described above. The ephrin-A2 Y→F mutant was made using the primer pairs A2F (5'
CGGAGAAAGTTCCAGCTCTACAACGCCCTTCTCCCTGGGCTTCGAGTTC 3')
and A3R (5'
GAACTCGAAGCCCAGGGAGAAGGGCGTGTAGAGCTGGAACCTTCTCCG 3').

The ephrin-A3 F→Y mutant was made using the primer pairs A3F (5' CTCGGAGAAGTTCCAGCGCTTCAGCGCCTTCTCTCTGGGCTACG 3') and A3R (5' CGTAGCCCAGAGAGAAGGCGCTGAAGCGCTGGAACTTCTCCGAG 3')

Western blot and Syncytia. To determine expression of the CedPV F and G genes, HeLa-USU cells in 25cm² flasks were transfected with a 1:3 ratio of F to G (total 2μg) using LipofectamineTM LTX and PlusTM Reagent (Invitrogen) in serum-free Opti-MEMTM (Life Technologies, Grand Island, NY). After overnight expression cells were harvested, lysates were prepared and nuclei were removed with a centrifugation step. The lysates were immunoprecipitated for 1 hour at room temperature using S-protein agarose beads (EMD Biosciences Inc., Madison, WI). The samples were then washed twice with lysis buffer (100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1% Triton X-100), and once with DOC buffer (100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS) as previously described[121], boiled in 4X NuPage® sample buffer (Life Technologies) containing 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and run on a NuPage® 4-12% Tris-acetate gel (Life technologies) and transferred to a nitrocellulose membrane. Expression was detected by western blot using horseradish peroxidase-conjugated rabbit anti-S-Tag polyclonal antibody (Bethyl Laboratories Inc., Montgomery, TX). To observe the functional activity of F and G glycoproteins, 293T cells were transfected as described, and after overnight expression syncytia were observed and photographed under brightfield conditions using an OlympusTM IX81 microscope connected to a Cooke CorporationTM SensiCamQE.

FC2 peptide design. A 36 amino acid residue fusion inhibiting heptad peptide sequence corresponding to the heptad repeat region 2 (HR2) of the CedPV F glycoprotein (CedPV-FC2) was designed based on Sable (<http://sable.cchmc.org/>), University of Cincinnati, OH)) secondary structure analysis of CedPV F and alignment of the CeV F glycoprotein sequence to that of HeV and NiV F. CedPV-FC2 peptide was synthesized by New England Peptide™ (Gardner, MA) (Ac-KVDLSNEINKMNQSLKDSIFYLREAKRILDSVNISL-amide). As a negative control for cell-cell fusion inhibition assays, a scrambled version of the above sequence was also prepared (scCedPV FC2) (Ac-RISKIDLKSNVDMILALSNRSDYQLNIFESVKNELK-amide). Each peptide was resuspended at a 50 µM concentration in dimethylsulfoxide (DMSO) (ATCC, Manassas, VA), then diluted to a 1 µM stock in PBS. At the highest concentration of peptide tested, the final amount of DMSO in each sample was 0.1%.

Cell-Cell Fusion Assay. Viral glycoprotein-mediated cell-cell fusion assays were carried out essentially as previously described [101], and as detailed in the methods for chapter 2. For these experiments, effector HeLa-USU cells were co-transfected with a 750ng of CedPV F plasmid and 2250 of CedPV G plasmid giving a 1:3 ratio of viral F and G genes and a total of 3µg of DNA using Lipofectamine™ LTX and Plus™ Reagent in serum-free Opti-MEM™ (Life Technologies). Transfections of HeV and NiV F and G plasmids were carried out under the same conditions. Target cells were prepared by seeding HeLa-ATCC or 293T cells known to express both ephrin-B2 and -B3, or by transfecting HeLa-USU cells with recombinant ephrin genes on a plasmid. Each fusion experiment was repeated at least three times.

To measure fusion inhibition by the CedPV FC2 peptides, the fusion assay was carried out as described above, with the addition of varying amounts of peptide or vehicle to the effector cells immediately preceding the addition of target cells.

3.4 Results

Functional expression of the Cedar virus F and G proteins. The open reading frames of the attachment (G) and fusion (F) glycoproteins were codon optimized and synthesized by Origene™ were subcloned into the pcDNA+hygro+CMV expression vector. By site directed mutagenesis an S-peptide tag (N-KETAAAKFERQHMDs-C) was added to the 3' end of the F open reading frame (corresponding to the C-terminus and cytoplasmic tail of the F glycoprotein) and to the 5' end of the G open reading frame (the N-terminus of the G glycoprotein), to allow detection of expression. HeLa-USU cells were transfected with 2 µg each of CedPV F or CedPV G, or with a plasmid encoding a non-S-tagged protein as a negative control. 24 hours after transfection lysates were prepared and expression was detected by immunoprecipitation with S-protein agarose beads (Novagen). CedPV F and G expression were detected by NuPage® (Invitrogen) gel electrophoresis followed by Western blotting using HRP-conjugated rabbit anti-S-peptide polyclonal antibody. Expression of CedPV G was robust and resulted in detection of a single band corresponding to the expected molecular weight of 72 kDa. The F glycoprotein was also expressed and was proteolytically cleaved as determined by the presence of an approximately 65 kDa band representing the uncleaved F₀ (predicted molecular weight 64.79 kDa), as well as the detection of a second band of approximately 52 kDa, most likely representing the C-terminal F₁ portion of the cleaved glycoprotein. The N-terminal F₂ portion was not detected due to the C-terminal S-peptide tag being removed along with

the F₁ glycoprotein segment. An additional band of a slightly higher molecular weight than the F₁ band was also detected. Since this additional band is faint and only accounts for a very small relative portion of the total glycoprotein, it is likely an incompletely cleaved product, or the product of an alternate, less favored cleavage site (**Fig. 8**). Since the F glycoprotein was proteolytically cleaved and thus likely activated for fusion, we sought to determine if the F and G glycoproteins expressed in this system were functional and capable of mediating membrane fusion. To determine this, effector cells were prepared by co-transfection of the F and G open reading frames of CedPV, or NiV as a positive control, in HeLa-USU cells which were then infected with vTF7.3, and target cells were prepared by infecting ephrin-B2 negative HeLa-USU cells and ephrin-B2 positive 293T cells with vCB21R. Mixing of the 293T target cells with the viral glycoprotein expressing effector cells resulted in syncytia formation (**Fig. 9**), indicating that the recombinant CedPV F and G glycoproteins were functional when expressed in this system and capable of mediating membrane fusion in mammalian cells. These effector and target cell populations were also processed to quantitate the fusion activity observed by measuring the β -galactosidase activity.

Figure 8. Expression of recombinant CedPV F and G glycoproteins.

The open reading frames of CedPV F and G were codon optimized, synthesized, and subcloned into an expression vector. An S-peptide tag for detection was added and the constructs were used to transfect HeLa-USU cells. 48 hours after transfection lysates were prepared and precipitated using S-protein agarose beads. The lysates were separated by 4-12% NuPAGE gel electrophoresis and visualized by Western Blot using HRP-conjugated rabbit anti-S-peptide polyclonal antibody.

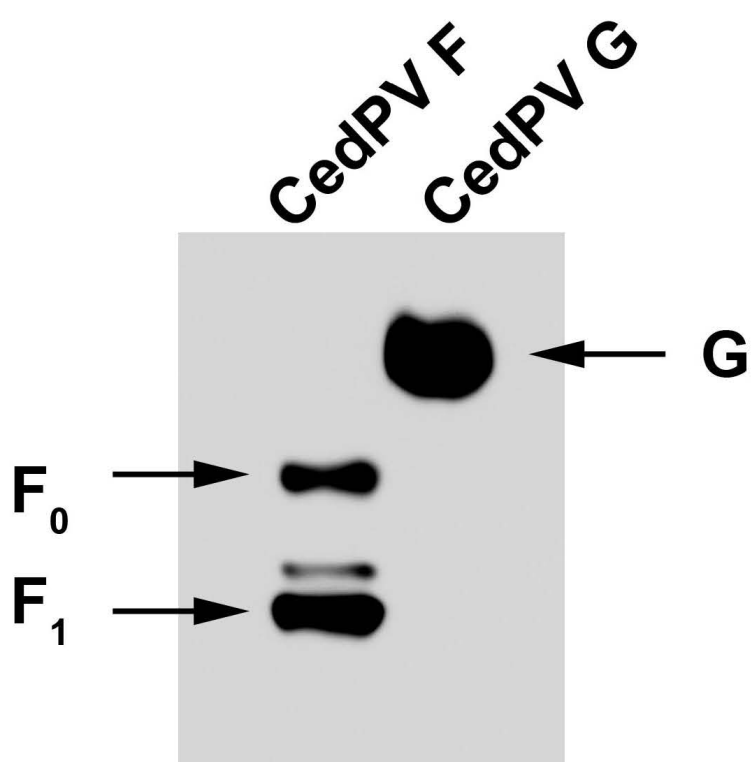


Figure 9. Functional expression of CedPV F and G results in syncytia formation.

HeLa-USU effector cells were cotransfected with a 1:3 ratio of CedPV or NiV F and G and infected with vTF7.3. HeLa-USU or 293T target cells were infected with vCB21R. The two cell populations were mixed at a 1:1 ratio in wells of a 96-well plate resulting 2×10^5 cell per well in a volume of 0.2ml. After 3 hours at 37°C, the cells were photographed at 400X magnification.

Syncytia

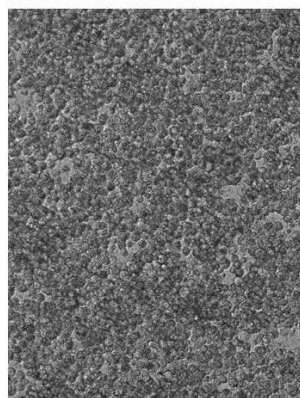
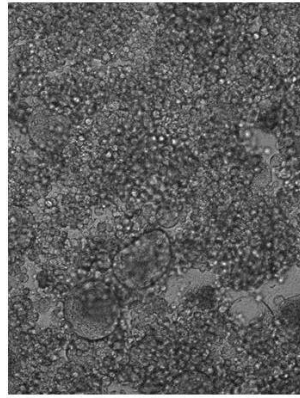
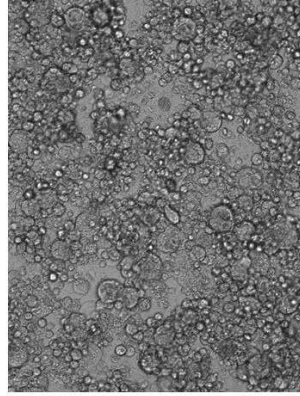
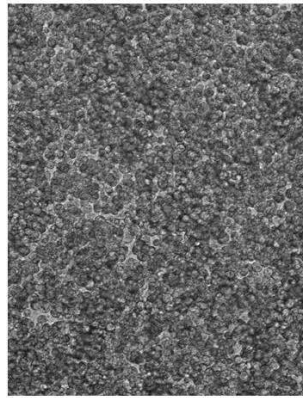
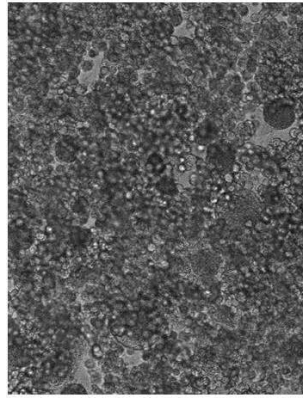
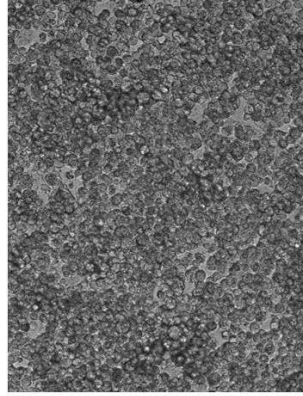
CedPV F and G

NiV F and G

Vector only

HeLa
USU

293T



Quantitation of Cedar virus glycoprotein mediated cell-cell fusion activity. The CedPV mediated cell-cell fusion activity observed in the syncytia formation assay was quantitated using the β -galactosidase reporter gene assay to confirm the use of ephrin-B2, but not ephrin-B3 as functional receptors for CedPV [114]. Here, the CedPV F and G were either each transfected singly, or co-transfected into ephrin-B2 and -B3 negative HeLa-USU cells. Fusion of these effector cells with target cells expressing neither ephrin-B2 nor -B3 (HeLa-USU cells), ephrin-B2 or ephrin-B3 alone (HeLa-USU-B2, HeLa-USU-B3 respectively), or a mixture of ephrin molecules including ephrin-B2, was evaluated at a neutral pH using the β -galactosidase reporter gene assay [101] (**Fig. 10**). Using this system it was determined that the CedPV envelope glycoproteins F and G, like the well characterized HeV and NiV envelope glycoproteins, can mediate cell-cell membrane fusion at a neutral pH and requires the co-expression of both the attachment (G) and fusion (F) glycoproteins. Additionally, it was determined that CedPV F and G co-expression is able to utilize ephrin-B2 as a fusion triggering receptor, but unable to utilize ephrin-B3, confirming the initial observations made with live virus [114].

Specificity of Cedar virus envelope glycoprotein-mediated cell-cell fusion. To demonstrate the specificity of CedPV glycoprotein-mediated fusion assays we designed a 36 amino acid fusion inhibitory heptad peptide (CedFC2) corresponding to the HRB domain of the CedPV F glycoprotein. It has been well documented for many viruses, including HeV and NiV [61, 100, 120] that peptide fusion inhibitors to class I viral fusion proteins that mediate membrane fusion at neutral pH are potent inhibitors of fusion activity. In the presence of nanomolar amounts of CedFC2, CedPV glycoprotein-mediated fusion was significantly inhibited, while the presence of a scrambled form of

the peptide, scCedFC2, had little inhibitory effect in the cell-cell fusion assay (**Fig. 11**). These data confirm the specificity of the cell-cell fusion assay activity. Additionally, the presence of NiVFC2, a fusion inhibiting heptad peptide previously described [61] capable of blocking both HeV and NiV glycoprotein-mediated fusion, also possessed some inhibitory activity against CedPV mediated cell-cell fusion (**Fig. 12**).

Figure 10. Quantification of CedPV glycoprotein-mediated cell-cell fusion.

HeLa-USU effector cells were transfected with empty vector as a negative control, the F and G glycoproteins of CedPV, HeV, or NiV, or CedPV F or G alone. Effector cells were infected with vaccinia virus vTF7.3 containing a T7 polymerase gene. Target HeLa-USU, HeLa-USU-Br, HeLa-USU-B3, or 293T cells were infected with vaccinia virus vCB21R containing the *E. coli lacZ* cassette under a T7 promoter. Effector cells were mixed with target cells and allowed to fuse for 2.5 hours, after which the cells were lysed and fusion rates were measured as rates of β -galactosidase activity.

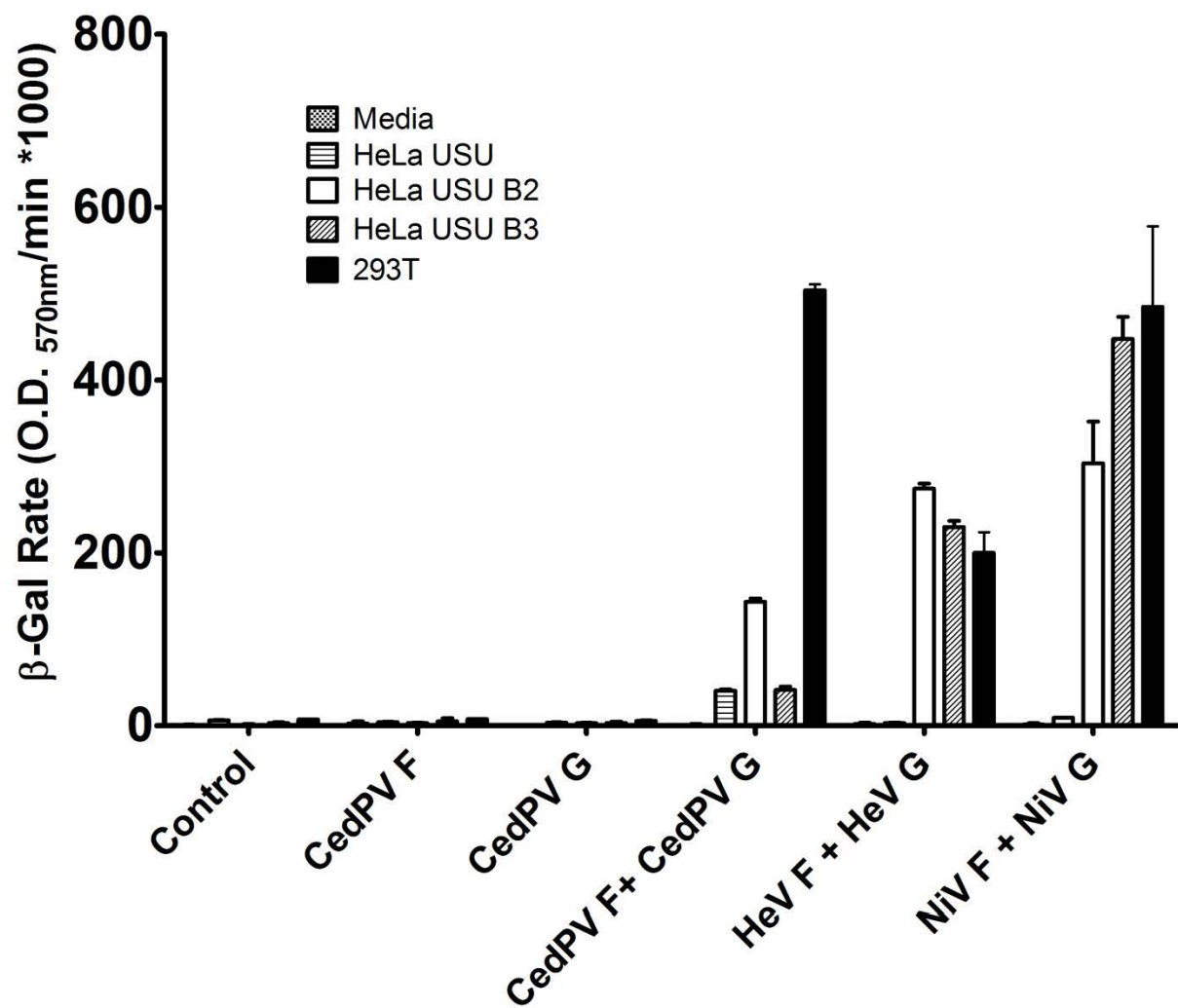
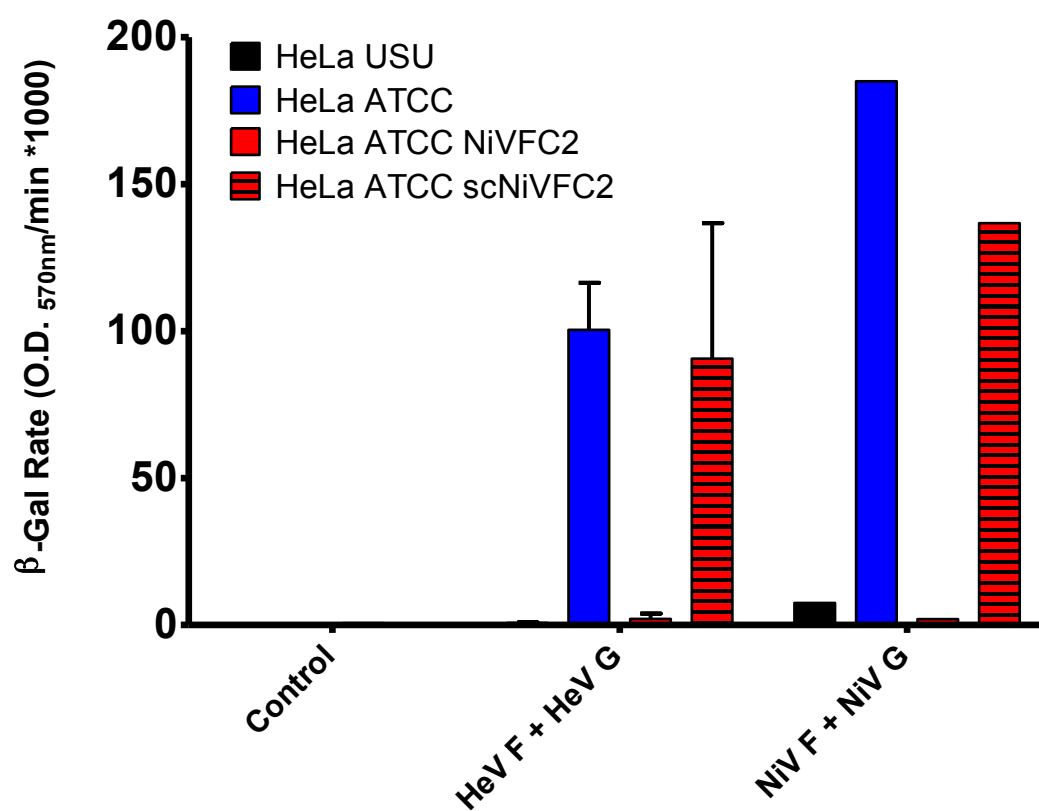


Figure 11. Inhibition of CedPV fusion by NiV F sequence-derived FC2 peptides.

Effector cells expressing CedPV, HeV, and NiV F and G were infected with vTF7.3, and target HeLa-ATCC cells were infected with vCB21R. Immediately prior to mixing of the cell populations, the NiV F HRB derived peptide NiVFC2, or a scrambled version of the peptide, scNiVFC2 was added to effector cells. HeLa-ATCC target cells were added and after 2.5 hours at 37°C, the cells were lysed and β -galactosidase activity was quantified.

A



B

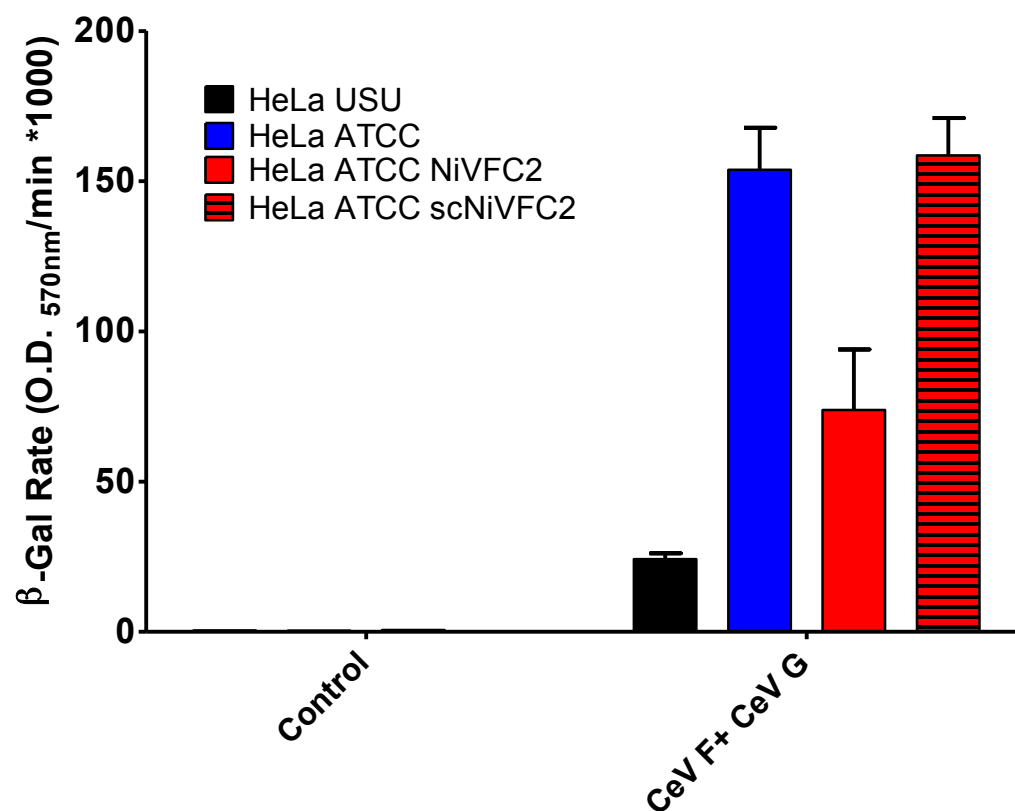


Figure 12. Specificity of CedPV glycoprotein-mediated cell-cell fusion.

CedPV, HeV, and NiV F and G expressing effector cells were prepared as described and infected with vTF7.3. Immediately prior to mixing with vCB21R infected HeLa-ATCC target cells, dilutions of CedPV F HRB-derived peptides were added to the effector cells. As controls, scrambled versions of the peptides were also added (scCedFC2). After fusion for 2.5 hours at 31°C, cells were lysed and β -galactosidase activity was quantitated.

Ephrin receptor tropism of Cedar virus. It was y interesting that Cedar virus was able to fuse with and enter cells using ephrin-B2, a henipavirus receptor, despite a relatively low level of homology between the CedPV attachment glycoprotein and those of Hendra virus (29%) and Nipah virus (30%), leading us to test the ability of CedPV G to bind the full panel of ephrins. Recombinant soluble fc-tagged human ephrins (R&D Systems, Minneapolis, MN) were used to co-immunoprecipitate soluble CedPV G, and binding was qualitatively assessed by Coomassie staining. It was found that CedPV is able to bind and co-precipitate ephrin-B1 as well as -B2, and surprisingly, was also able to bind the A-type ephrins: ephrin-A1, -A2, and -A5 (K. Xu, Memorial Sloan-Kettering Cancer Center, New York, NY).

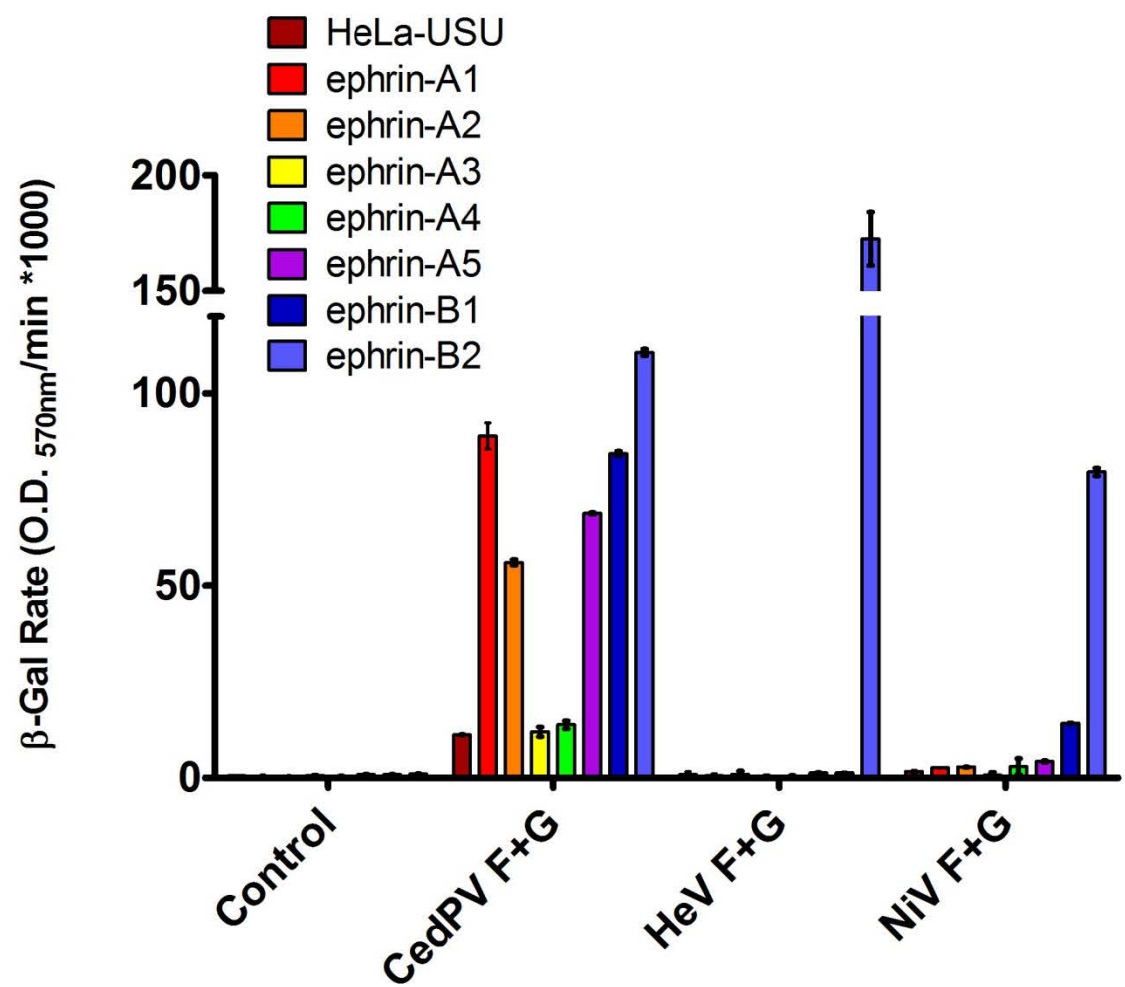
To determine if the binding interactions of CedPV G with ephrins -B1, -A1, -A2, and -A5 were functional in triggering membrane fusion mediated by the CedPV F and G envelope glycoproteins, a series of ephrin receptor target cells were prepared by transient transfection of HeLa-USU cells with expression plasmids encoding ephrins -B1, -B2, -A1, -A2, and -A5, and these target cell populations were then used in the cell-cell fusion reporter gene assay with CedPV effort cells bearing the F and G envelope glycoproteins. Remarkably, CedPV was demonstrated capable of not only utilizing and fusing with the transmembrane proteins ephrin-B1 and ephrin-B2, but was also able to employ the GPI-anchored ephrin-A1, ephrin-A2, and ephrin-A5 proteins (**Fig. 13**). The ability of CedPV to use such an expanded set of functional ephrins to trigger its glycoprotein-mediated fusion activity was remarkable, and it was particularly interesting because both transmembrane anchored ephrin-B1 and -B2, and GPI-anchored ephrin-A1, -A2, and -A5 are used. The implications of this on the triggering mechanisms of henipavirus fusion

and entry have yet to be explored. With this very broad receptor tropism, it also suggests that CedPV may have an even broader cellular and/or species tropism in comparison to HeV and NiV. This promiscuous tropism could also enhance the transmission and maintenance of CedPV within bat colonies..

Figure 13. Ephrin receptor tropism of CedPV.

Effector cells were prepared by transfection of the F and G open reading frames of CedPV, HeV, or NiV, and infected with vTF7.3. Target cells were prepared by transfection of HeLa-ATCC cells with ephrins-A1-A5, -B1, and -B2 as a control.

Untransfected HeLa-USU cells were included as a negative control. The target cells were infected with vCB21R, and mixed with effector cells in duplicate wells of a 96-well plate. Following a 2.5-hour fusion period, the cells were lysed and β -galactosidase activity was measured.



3.4 Discussion & future directions

Cedar Virus mediated membrane fusion. We have demonstrated the functional expression of the CedPV attachment (G) and fusion (F) glycoproteins, and the membrane fusion is pH independent and requires the presence of both the F and G glycoproteins. Additionally, it was shown that CedPV possesses a remarkably broad ephrin receptor tropism, able to utilize not only the transmembrane anchored henipavirus receptor ephrin-B2, but also ephrin-B1, as well as the GPI-anchored ephrins-A1, -A2, and -A5. This is first description of a paramyxovirus which is able to utilize both a membrane anchored protein receptor and a GPI-anchored protein receptor.

GPI-anchored proteins as viral receptors. CedPV is one of only a few viruses described to date that are able to use GPI-anchored proteins for pH-independent membrane fusion activation at the cell surface. GPI-anchored proteins have been shown to play a role in the entry of many different viruses, but generally only as far as they are prominent components of lipid rafts (reviewed in ref. [122]). Decay-accelerating factor (DAF) is a GPI-anchored protein that has been shown to be required for the cellular entry of some enteroviruses, including various echovirus serotypes [123], however, the binding of echoviruses to DAF at the apical surface of polarized epithelial cells in culture leads to the transport of the virus to tight junctions and subsequent endocytosis likely due to cross-linking of DAF [124], which is entirely different from the direct fusion of CedPV at the cell membrane. One isoform of avian Tumor Virus receptor A (TVA) which is used as a receptor by avian sarcoma and leukosis virus is GPI-anchored [125]. Two retroviruses, Mouse IAPV Endogenous Retrovirus and Jaagsiekte Sheep Retrovirus

(JSRV) have been found to infect cells using GPI-anchored proteins. JSRV infects cells using HYAL2 as a receptor [126]. Mouse IAPV endogenous retrovirus, interestingly, can enter cells using any of the five GPI-anchored A-type ephrins, sharing three receptors with CedPV. However, IAPV is unable to use any of the trans-membrane B-type ephrins [127]. Interestingly, in work done by Dawanniuex and colleagues [127], the presence of ephrin-A2 and ephrin-A5 was demonstrated in the oocytes and spermatozoa of mice, respectively, indicating that if this is true of bats as well, it may indicate an additional method of virus spread in the Pteropid population. To the best of our knowledge, CedPV is the first example of a paramyxovirus which is capable of using both transmembrane proteins and GPI-anchored proteins as cellular receptors.

The G glycoprotein and ephrin receptors: lock, key, and latch. The interactions of the henipavirus G glycoproteins with ephrin-B2 and -B3 were recently reviewed by Steffen et al., and have been shown to occur by the insertion of a flexible, solvent exposed loop of ephrin-B2/B3 into the hydrophobic core of the G glycoprotein. The B2/B3 G-H loop consists of six amino acids (F/Y_{117/120}SPNLW_{122/125}) which are thought to fit into the binding pocket of G by an induced-fit lock and key mechanism, which is stabilized by a conformational shift of W_{122/125}, serving to “latch” the loop in place [128]

The observation that mutation of W504 and E505 of HeV/NiV specifically disrupts interaction with ephrin-B3, while mutation of E533 disrupts both ephrin-B2 and B3 interaction [71, 129] is supported by the fact that E533, but not W504 and E505 are conserved in CedPV G, which does not bind ephrin-B3. The functional consequences of the sequences changes in CedPV G have yet to be evaluated, and it is possible that mutagenesis of CedPV G will reveal additional ephrin contacting sites mediating the

observed broad ephrin subtype interactions, and could shed more light on the interactions of HeV and NiV with ephrin-B2 and -B3. It has been suggested that the only major structural rearrangement of the ephrin molecules upon G engagement is in the G-H loop, specifically in the residue W 125, which is hypothesized to form a “latch”, strengthening the interaction between G and ephrin-B2/B3 [128]. This “latch” mechanism does not appear to be applicable to the binding of ephrins by CedPV G, as it cannot bind ephrin-B3 despite the presence of the “latching” W residue, but can bind ephrins -B1, -A1, -A2, and -A5, which do not possess a W residue in the G-H loop, but instead have a M (ephrin-B1), or a L (ephrins-A1, -A2, -A5) (**Table 2**).

There are four residues in HeV and NiV G which have been shown to interact with the L residue in the G-H loop of ephrin-B2/B3, and four which have been shown to contact the W residue, one of which, W504, overlaps and interacts with both L and W, and two of which (E505 and G506) also contact the P residue. Of these 8 residues, only one (G506) is conserved in CedPV G, which is in line with the observed receptor tropism of CedPV G, as the 5 fusion mediating ephrins-B1, -B2, -A1, -A2, and -A5, have four different amino acid pairs at that location (ephrin-B1-YM, -B2-LW, -A1-IL, -A2 and -A5-SL). This indicates that the interaction between CedPV G and its ephrin receptors is somewhat different than the interaction between HeV G/NiV G and ephrin-B2/B3.) Four of the nine residues of HeV and NiV G which have been demonstrated to contact the F/Y residue in the G-H loop of ephrin-B2/B3 are different in CedPV G, as are four of the nine P contacting residues. Overall, with three G residues contacting more

Table 2. Ephrin G-H loop core sequences and CedPV fusion.

	G-H Loop Sequence	CedPV Fusion	HeV Fusion	NiV Fusion
Ephrin-A1	FTPFIL	+	-	-
Ephrin-A2	FTPFSL	+	-	-
Ephrin-A3	YSAFSL	-	-	-
Ephrin-A4	YTPFPL	-	-	-
Ephrin-A5	FTPFSL	+	-	-
Ephrin-B1	FSPNYM	+	-	-
Ephrin-B2	FSPNLW	+	+	+
Ephrin-B3	YSPNLW	-	+	+

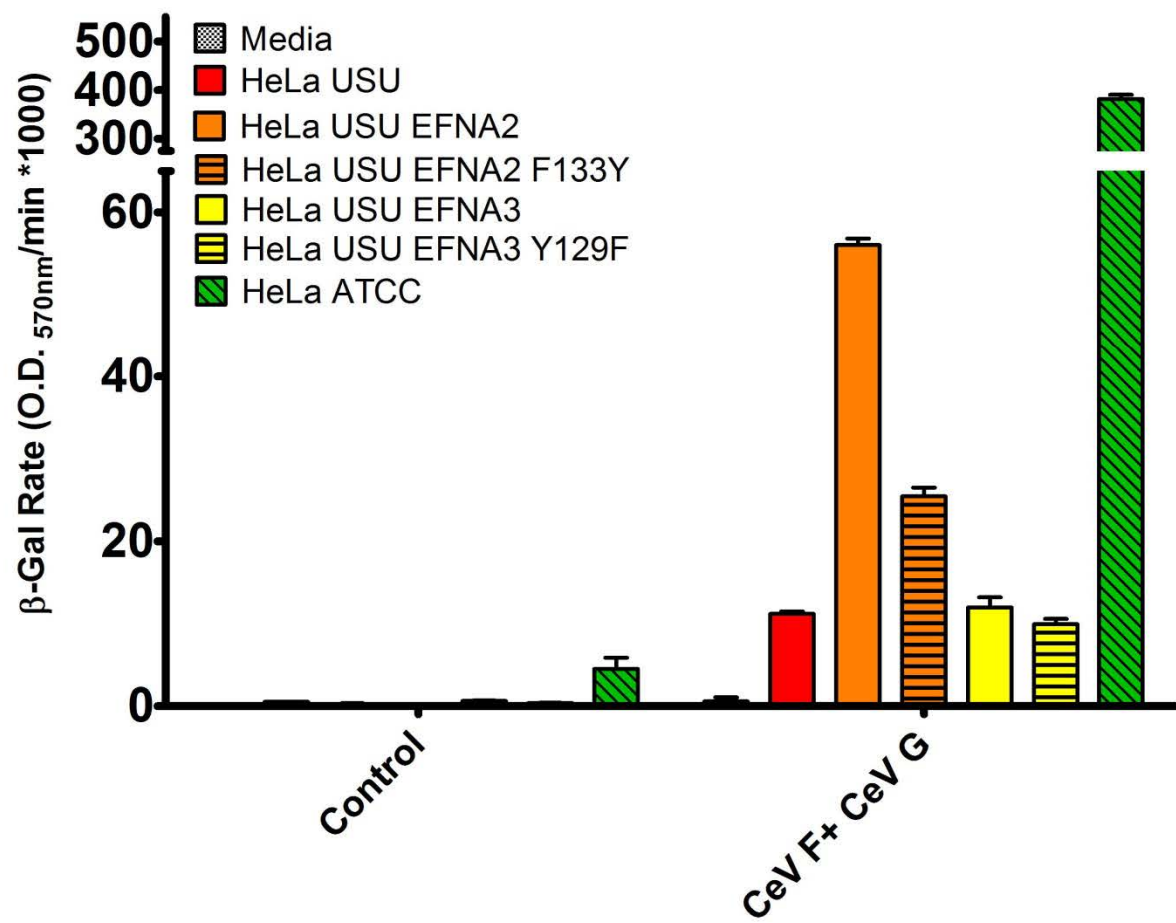
than one ephrin G-H loop amino acids, 14 out of 26 ephrin contacting residues in HeV/NiV G are not conserved in CedPV G.

The only residue in the G-H loop of the ephrin molecules that is uniquely conserved in the CedPV G interacting ephrins is the F at the beginning of the G-H loop. The non-CedPV G interacting ephrins all carry a Y at that location. To determine if this F residue is critical for CedPV G receptor functionality, the F residue in the fusion-promoting ephrin A2 was mutated to Y, and the Y residue in the non-fusion promoting ephrin-A3 was mutated to F. The mutants were transfected into HeLa-USU cells and their ability to fuse with CedPV F+G expressing effector cells was evaluated. It was found that the F to Y mutation in ephrin-A3 decreased its fusion promoting activity by about 50%.

Interestingly, the reciprocal mutation of Y to F in ephrin A3 did not have any fusion enhancing effect, indicating that an F in this position in the context of ephrin-A2 is fusion enhancing, but that it is not sufficient to enhance fusion in the sequence context of ephrin-A3. However, this work is preliminary, and cannot be interpreted with confidence until equal surface expression of the wild-type and mutant ephrins has been demonstrated. The only other difference in the G-H loop of ephrin-A2 and -A3 is a P in residue -A2 and an A in A3 residue at the 3rd position of the loop. Ephrin-A2 F→ Y, P→ A and ephrin-A3 Y→ F, A→ P double mutants have been constructed and will be evaluated in fusion assays.

Figure 14. Effect of mutation of G-H loop of ephrin-A2 and ephrin-A3 on CedPV glycoprotein mediated cell-cell fusion.

Effector and target cells were prepared as described in the legend to **Figure 13** except target cells were transfected with wild-type ephrin-A2, ephrin-A2 F133Y, wild-type ephrin-A3, or ephrin-A3 Y129F. The cell populations were mixed and allowed to fuse for 2.5 hours at 37°C. Fusion activity was quantified as the rate of β -galactosidase activity.

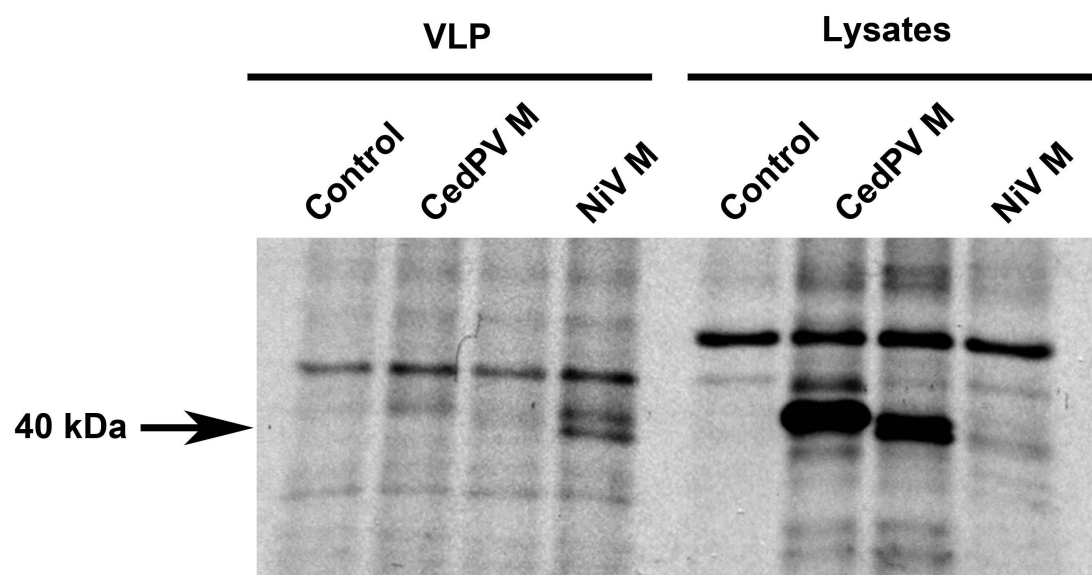


New research directions offered by Cedar Virus. The observations discussed above provides a foundation for a wide variety of new research directions for both investigating the basic cell biology of CedPV including a large tool set to explore the details of CedPV envelope glycoprotein mediated membrane fusion and the details of its protein receptor triggering processes. These platforms will also be of particular interest in further exploring similar areas of HeV and NiV biology as well. .

Work has already begun to characterize the matrix (M) protein of CedPV, which appears to differ from that of HeV and NiV in that it is unable to mediate VLP formation and budding by itself. Indeed, amino acid alignments of CedPV M with HeV M and NiV M have shown an imperfect conservation of L-domain sequences. Based upon alignments of CedPV M with the M proteins of HeV and NiV, two putative L-domains have been identified: ₇₁YQYM₇₅, and ₁₀₁FPLGVG₁₀₆. These sequences are similar but not identical the established NiV L-domains ₆₂YMYL₆₅ and ₉₂YPLGVG₉₇. These putative L-domain-like sequences of CedPV differ from the L-domains of NiV by only three amino acids. Preliminary mutagenesis studies modifying the L-domain-like sequences of CedPV M and conversion to those of NiV M have been unable to enhance CedPV M-mediated budding of VLPs, suggesting that CedPV may require the presence of other viral proteins to efficiently bud (**Fig. 15**). Further preliminary experiments where the co-expressing the F glycoprotein of CedPV together with the M protein have also been unable to enhance M release. The cellular or viral factors mediating CedPV particle formation and budding are still being investigated.

Figure 15. CedPV M mediated virus-like particle production.

293T cells were transfected with NiV M, CedPV M, or empty vector as a control and radiolabeled with ³⁵S methionine and cysteine. 18 hours after applying label, M proteins were immunoprecipitated from the lysates, and VLPs were harvested from the supernatants. The VLPs and lysates were run on an SDS-PAGE gel and visualized by autoradiography, with the M proteins appearing as a band at approximately 40 kDa.



It is also not yet known if the broad ephrin tropism of CedPV displayed in present cell-cell fusion assays *in vitro* translates to a broader cell or species tropism displayed by live virus. Determining if live CedPV is able to infect cells using the GPI-anchored A-type ephrins will be particularly interesting, and will be crucial to establishing the biological relevance of the results of the present work. One way to address this question is to establish HeLa-USU based cell-lines expressing the CedPV fusion competent ephrin-A1 and the fusion non-permissive ephrin-A3 and use them for live virus infection studies at the AAHL, CSIRO, which is presently the only location of infectious CedPV, and because CedPV was isolated in the BSL-4 laboratory, its use will not be permitted at any lower containment level. However, an alternate approach that to study viral particle entry could be pursued using one or more pseudotyped viral particle assays using the CedPV F and G glycoproteins. One example makes use of a vesicular stomatitis virus (VSV) backbone-based system using the Indiana strain, whereby the VSV G glycoprotein ORF has been deleted, and a reporter gene is inserted, such as a form of secreted alkaline phosphatase. Upon entry of the pseudotyped viral particle into a susceptible cell, the production of alkaline phosphatase can be quantitated and used as a measure of entry [130]. A second platform is a well described HIV-1 backbone-based pseudotyped viral system that has been successfully adapted to the study of NiV [131]. In this system, the HIV-1 envelope glycoprotein has been deleted from the backbone of virus on a plasmid, and a luciferase reporter gene has been added to form the plasmid pNL4-3-Luc-E-R⁺ [132]. Entry of a functional pseudotyped viral particle into a susceptible cell as measured

by luciferase gene activity can be assayed by measuring conversion of a luciferase substrate on a luminometer.

It will also be important to quantitate the relative affinities of CedPV G for the various ephrin molecules tested here in the cell-cell fusion assays. A Biacore analysis, which may be particularly useful, is a process by which the affinity of binding of a pair of molecules is determined by immobilizing one member of the pair on a glass sensor chip covered with a thin layer of gold, and flowing varying concentrations of its binding partner over the chip at a steady rate in a buffer. Polarized light is then shone onto the glass surface forming an electric field, which is absorbed by electron clouds in the layer of gold. The absorption of this energy by the gold layer results in the formation of plasmons, waves of electron charge density, which ultimately results in a reduction of the amount of light which is reflected back to the sensor. The refractive index of the buffer system determines the degree to which the polarized light is absorbed by altering the resonance of the plasmons between the gold layer and the buffer. This is known as surface plasmon resonance (SPR) and can generally be recorded in this system as the angle at which the reflection of light reaches a minimum. Binding of its partner by its ligand in the buffer system changes the refractive index and thereby alters the SPR, which is detected as a change in the angle of minimal reflection (reviewed [133] and [134]. The BIAcore system can be used to study the affinity of interactions of numerous and varied types of biomolecules, and has been applied to the study of viral glycoproteins and their receptors [135]. We are currently examining these assays using a soluble tetrameric form of CedPV G designed, produced and purified by Deborah Fusco (USUHS) which, along with a full panel of A and B-type soluble FC-tagged ephrins

(R&D). . Given that CedPV has consistently shown higher rates of fusion with ephrin-A1 as a surface receptor than with ephrin-A2, these experiments could allow us to determine if higher G-receptor affinity translates to increased rates of fusion, which could have implications for the requirement of a continual G-receptor-F association for initiation and completion of the fusion process. If it is true that continual engagement of receptor by G is required for G to chaperone F through the conformational changes required to mediate membrane fusion as proposed by Porotto and colleagues [68], then it stands to reason that a higher affinity interaction of G with receptor might translate to an increased rate of fusion.

The exposed G-H loop has been proposed as a potential target of antibody- or small-molecule-based anticancer drugs [136]. If the interaction of CedPV G with ephrin-A5 is of high affinity, it is possible that CedPV G could serve as a platform for the development of such drugs.

The increasing awareness of the potential threats posed by henipaviruses, particularly following the demonstration of direct human-to-human transmission of NiV in Bangladesh, had stimulated an increase in global serosurveillance measures of bat populations, not just in Australia, Southeast Asia and the Indian subcontinent, but also in Africa [77] and has also sparked an interest among investigators to evaluate the risk of henipavirus presence or establishment in Europe [137, 138]. The presence of henipavirus infection by either serology or PCR has been described in a wide variety of bats, both Pteropid and non-Pteropid fruit bats, as well as insectivorous bats, in a large geographical area spanning from Papua New Guinea, over Australia, Southeast Asia, China, the Indian subcontinent, all the way to Madagascar and Western Africa. However, the isolation of

HeV or NiV has been described at a rate that is lower than might be expected given the serological data, indicating that there likely exist in fact previously unrecognized henipaviruses of unknown pathogenicity, which are circulating in geographically diverse Pteropid populations and cross react with HeV and NiV by commonly used PCR- or serum-based detection methods. Indeed, the discovery of CedPV, which appears to be the first new member of the genus *Henipavirus*, lends support to this possibility. It is not yet known if CedPV would be detected in any of the surveillance tests used to monitor HeV and NiV, but since CedPV shares an important receptor usage profile with HeV and NiV, it is clearly possible that certain structural epitopes in the attachment G glycoprotein might well be conserved among all three viruses. Together, these data highlight the increasing need to develop detection methods that are specific enough to distinguish the highly virulent NiV and HeV from other circulating “henipa-like” viruses. Here a variety of new tools are described with now offer the possibility of carrying out a detailed examination of the cross-reactive and cross-neutralizing epitopes of the HeV, NiV, and CedPV F and G glycoproteins, and also to employ CedPV as a new platform to study the cell biology and virological aspects of the henipaviruses outside of high level BSL-4 containment.

Chapter 4

Functional interactions between *Henipavirus* envelope glycoproteins

4.1 Introduction. Paramyxovirus fusion generally requires (with few exceptions) the presence of both the F and G glycoprotein, an interaction that is usually type-specific in that the F glycoprotein of one virus will generally not be able to functionally interact with the attachment glycoprotein of another. Only a limited number of paramyxoviruses have been found capable of mediating membrane fusion in any heterotypic combination. For example, it has been shown that the morbillivirus canine distemper virus (CDV) F is able to mediate cell-cell fusion in combination with the measles virus (MeV) attachment protein (H) another morbillivirus, however the level of cell fusion activity observed was low and the converse combination (CDV H and MeV F) was not functional [139].

Whereas the respiroviruses, Sendai virus and hPIV1 and the henipaviruses, HeV and NiV are the only known paramyxoviruses within the same genera that are capable of mediating efficient membrane fusion in bidirectional heterotypic combination [140] [100]. The precise domains within the glycoprotein pairs that are responsible for heterotypic activity of paramyxovirus fusion and attachment glycoproteins is unknown, but are likely conserved of structural features that exist between the different species of glycoproteins.

The extent of amino acid sequence identity between the CedPV F and G glycoproteins in comparison to those of HeV and NiV is much lower (42% and 43% for HeV and NiV G, 29% and 30% for HeV and NiV F) as that which exists between HeV and NiV. However, based on hydrophobicity plots and secondary structure predictions, the basic structural features between them appear conserved. Also some detail on conserved elements are known, for example, two residues (D257 and D260), which are

thought to be important for the interaction between the globular heads of G monomers forming the homodimer of HeV G [64, 71], are conserved in CedPV G. In addition, the series of 9 leucine residues within the stalk domain of HeV G that appear critical in stabilizing the conformation of G in a manner required for proper interaction with F and subsequent triggering of fusion [35], are also well conserved in CedPV G. Four isoleucine residues are replaced with leucine residues in CedPV G, a substitution that is also conserved and likely allows for retention of important structural and functional features. There is a substitution of I131 to a T residue, but since isoleucine and threonine are also structurally similar, this is also likely to allow for retention of its role in the protein. The conservation of these residues highlights the likely structural conservation of the CedPV, NiV and HeV attachment G glycoproteins, despite an overall low level of primary amino acid sequence homology. Thus, the suggestion of whether CedPV glycoproteins, particularly G, could serve as a functional heterotypic partner with HeV and/or NiV F is an intriguing possibility and worthy of testing.

4.2 Aim and Hypothesis.

Specific Aim 1: Determine if the F and G glycoproteins of CedPV exhibit heterotypic functionality when co-expressed with the F and G glycoproteins of HeV and NiV.

Hypothesis: Based on the high level of predicted structural similarity between the glycoproteins of HeV and NiV to that of CedPV, we hypothesize that the F and G glycoproteins of CedPV will exhibit an ability to function in mediating membrane fusion in heterotypic combination with the F and G glycoproteins of HeV and NiV.

4.3 Materials and Methods

Henipavirus envelope glycoprotein-mediated cell-cell fusion assay. The viral glycoprotein mediated cell-cell fusion assay was carried out as described in the methods section of Chapter 2. The envelope glycoproteins of CedPV, HeV, and NiV were co-transfected at a constant ratio of 1:3 F:G with a total DNA amount of 3 μ g (750ng F and 2,250 ng G), either in homotypic or heterotypic combination, and allowed to fuse with ephrin-B2-USU cells, ephrin-B3-USU cells, HeLa-USU, or HeLa-ATCC cells. Fusion rates were calculated as the rate of β -galactosidase activity. Each assay was conducted in duplicate and repeated at least three times.

4.4 Results

Heterotypic envelope glycoprotein functionality. As detailed previously in Chapter 3, CedPV glycoprotein-mediated membrane fusion was found comparable to that of HeV and NiV with target cells expressing the viral receptor ephrin-B2, and requiring the presence of both the F and G glycoproteins (**Fig. 10**). Previous work demonstrated that the F and G glycoproteins of HeV and NiV are able to mediate cell-cell fusion in bidirectional heterotypic combinations; meaning that the HeV F glycoprotein is able to mediate fusion when combined with the G glycoprotein of NiV, and the complementary combination (NiV F with HeV G) was also functional [100].

Given the relatedness of CedPV to the other known henipaviruses, and examination of CedPV F and G to test their ability to function in heterotypic combination with the F and G glycoproteins of HeV and NiV, was important and may be able to help detail the protein-protein interactions that are critical in the membrane fusion process. To address

this question, the results of Bossart et al [100] were first confirmed by conducting a homotypic and heterotypic henipavirus glycoprotein mediated cell-cell fusion assay. The F and G glycoproteins of HeV and NiV were co-transfected in homotypic (e.g. HeV F + HeV G) and heterotypic (e.g. HeV F + NiV G) combinations with a ratio of 1(F):3(G) and evaluated in the reporter gene cell-cell fusion assay described earlier using HeLa-ATCC, HeLa-USU, HeLa-USU-B2, or HeLa-USU-B3 as various target cell populations. As expected, the F and G glycoproteins of HeV and NiV were capable of mediating efficient cell-cell fusion in bidirectional heterotypic combinations, as was observed by Bossart et al [100].

The ability of CedPV F to mediate cell-cell fusion in combination with the G glycoproteins of HeV and NiV was examined next. Again, HeLa-USU effector cells were co-transfected with either the homotypic combination of CeV F + CeV G, or with the heterotypic combinations (CeV F + HeV G, CeV F + NiV G), and the reporter gene cell-cell fusion assay was carried out using HeLa-ATCC, HeLa-USU, HeLa-USU-B2, or HeLa-USU-B3 as the various target cell populations. We found that the F glycoprotein of CedPV was capable to functionally pair with the G glycoproteins of both HeV and NiV, indeed providing strong functional evidence in categorizing CedPV as the first new member of the genus *Henipavirus*. The fusogenic activities measured with effector cells expressing the heterotypic F and G combinations were not as great as those obtained from the cell-cell fusion reactions where the homotypic combinations were employed, which was similar to the previously reported data testing HeV-NiV heterotypic fusion activities [100]. Also, as predicted, the effector cells expressing heterotypic glycoprotein

combinations exhibited an ephrin receptor tropism based on the known characteristics of the G attachment glycoprotein.

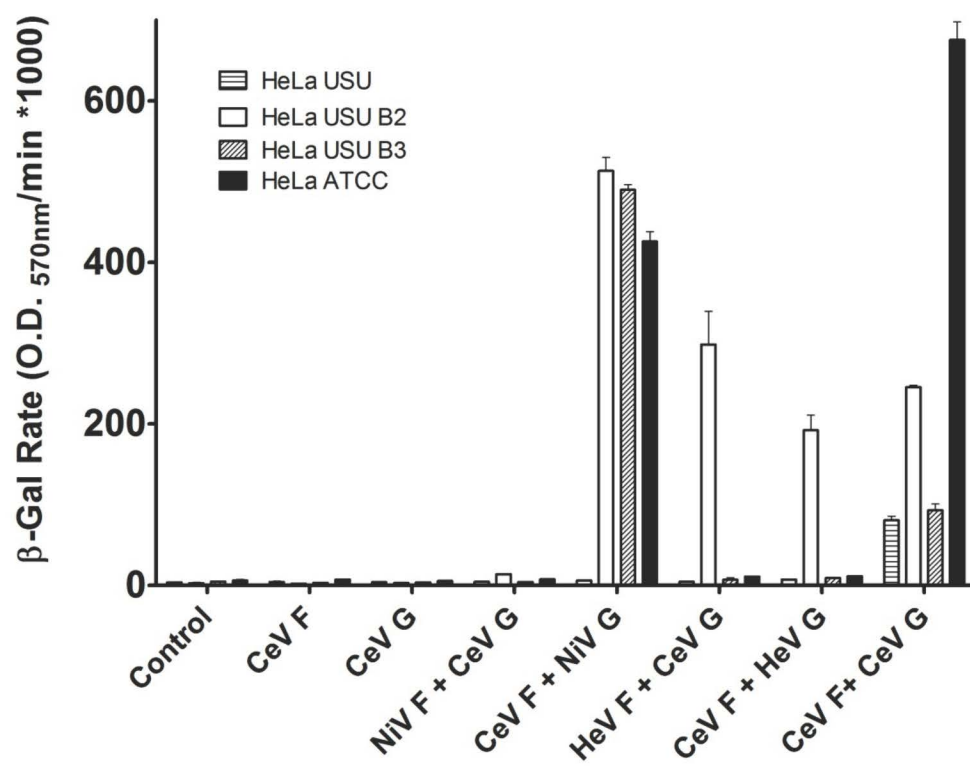
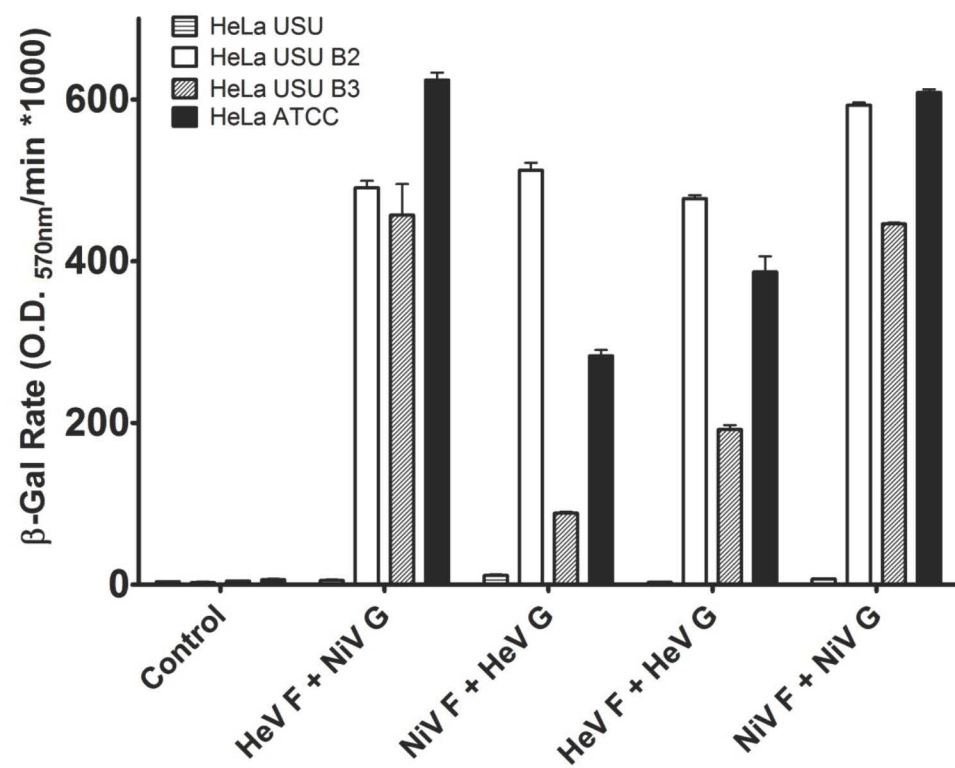
The converse experiment then examined the ability of CedPV G to functionally pair with the HeV and NiV F glycoprotein, and a similar reporter gene cell-cell fusion assay was carried out as before. Here, we found that the CedPV G was capable of supporting fusion promotion in heterotypic combination with HeV F, remarkably however it was found that , despite the high level of amino acid sequence homology between HeV F and NiV F, the combination of CedPV G with NiV F was not functional. Additionally, it was found that the tropism of the three functional heterotypic combinations were somewhat different. CedPV F in combination with NiV G was able to mediate efficient cell-cell fusion with HeLa-ATCC cells, the HeLa-USU-B2 cell line, and the HeLa-USU-B3 cell line, in agreement with the known receptor tropism of NiV G (**Fig. 16**). However, the glycoprotein combinations HeV F + CeV G and CeV F + HeV G were noted to be only functional with the HeLa-USU-B2 cell line. This observation was somewhat unexpected because HeV G is known to functionally engage both ephrin-B and -B3, however, the basis for this difference are as yet unknown.

Taken together, this is the first description of a functional cell-cell membrane fusion system in which the G glycoprotein of one henipavirus is functional in heterotypic combination with the F glycoprotein of one other member of the genus, but not with the F glycoprotein of another member. As mentioned earlier, given the high level of structural similarity of the F and G glycoproteins of CedPV, HeV, and NiV, the determinants of heterotypic interaction are likely to be specific protein domain or sequence related. This cell-cell fusion system now offers a novel opportunity to explore the nature of the

interaction between the F and G glycoproteins of the henipaviruses and can also potentially shed light on the paramyxovirus fusion process in general with particular focus on the fusion triggering mechanism by the ephrin protein receptors.

Figure 16. Heterotypic fusion activity of Henipavirus glycoproteins.

HeLa-USU effector cells were transfected with all nine possible combinations of the CedPV, HeV, and NiV F and G glycoproteins. They were infected with vTF7.3 and allowed to fuse with vCB21R infected HeLa-USU, HeLa-ATCC, HeLa-USU-B2 and HeLa-USU-B3 target cells in duplicate wells of a 96-well plate. After 2.5 hours, the cells were lysed and assayed for β -galactosidase activity. This experiment was repeated three times. For ease of interpretation, HeV/NiV heterotypic controls are presented in (A) and CedPV/HeV and CedPV/NiV heterotypic combinations are presented in (B).



4.5 Discussion and future directions

Taken together, the data presented here demonstrates that the CedPV envelope glycoproteins are able to mediate membrane fusion in imperfect bidirectional heterotypic combinations with envelope glycoproteins of HeV and NiV, providing strong functional evidence in support of the categorizing of CedPV within the genus *Henipavirus*.

Additionally, the heterotypic functionality of the CedPV F and G glycoproteins with those of HeV and NiV offer a unique platform to study the interactions of the henipavirus glycoproteins. For the first time a system is in place in which the G glycoprotein of one virus in a genus (CedPV G) is functional with the F glycoprotein of another member (HeV F) but non-functional with the G glycoprotein of another (NiV F). This system will afford a unique opportunity to carry out a variety of experimental approaches to further define the fusion triggering mechanism of the henipaviruses as well as paramyxoviruses in general. For example, heterotypic co-immunoprecipitation experiments could reveal if the observed lack of heterotypic function between NiV F and CedPV G is due to a lack of interaction between the glycoproteins, or if it is due to an imperfect interaction which impairs the transmission of the fusion promoting signal from G which triggers the fusogenic activity of F. Such experimental information will be crucial in detailing the F and G interaction features that are necessary for membrane fusion to occur. Further, if it is found that NiV F and CedPV G do interact, it will suggest that although receptor binding takes place a novel “uncoupling” of receptor binding and F protein triggering by G is at play. This could be a significant observation which may ultimately help reveal whether henipavirus fusion is regulated by a mechanism more in line with a “clamp” model versus “provocateur” model.

Determining the ephrin receptor tropism of the heterotypic CedPV F/HeV G and HeV F /CedPV G combinations could also address the question of how receptor binding by G triggers the fusogenic activity of F. It may be expected that the ephrin receptor tropism would be entirely dependent on the G glycoprotein, but since CedPV G is able to bind both transmembrane-anchored and GPI-anchored receptor proteins, it will be of interest to determine if CedPV G is able to trigger HeV F fusion activity via a GPI-anchored ephrin receptor.

This now expanded henipavirus glycoprotein fusion system could also be used to identify more precisely the critical domain(s) in the F glycoprotein which receives the fusion triggering signal from the G glycoprotein. For example, chimeric F glycoproteins could be constructed swapping various domains between HeV F and NiV F to determine which HeV F domains will impart heterotypic functionality to NiV F when in combination with CedPV G. If successful, detailed site-directed mutagenesis of specific amino acid residues may ultimately reveal critical residues required in F for fusion triggering, providing exceptional detail to the henipavirus fusion mechanism, which may be related to the specific domains of F required for its functional interaction with a partner G glycoprotein. Similarly, site-directed mutated constructs of the HeV and NiV G glycoproteins could be constructed and used to identify the precise residues which mediate the functional use of ephrin-B2 or ephrin-B3 as receptors. Both HeV and NiV F and G, in their native combinations, can mediate fusion using either ephrin-B2 or ephrin-B3- as receptor, whereas CedPV does not use ephrin-B3 (**Fig. 10** and [38, 114]). As expected, when CedPV F was paired with NiV G, both ephrin-B2 and ephrin-B3 expressing cells are permissive target cells for fusion. Unexpectedly however, when

CedPV F was combined with HeV G, only the ephrin-B2 cell line is able to mediate fusion, indicating that there is some underlying functional difference in the interaction of CedPV F with HeV G and NiV G in the context of receptor binding which ultimately influences the fusion triggering process. Chimeric HeV and NiV G glycoproteins could be constructed and then evaluated for ephrin-B2 and ephrin-B3 functional fusogenic tropism in heterotypic combination with CedPV F to determine which domain of NiV G can restore ephrin-B3 fusion triggering activity following binding, such as a particular role for either the globular head of G or the stalk domain.

Summary

The characterization of the attachment and fusion glycoproteins of CedPV, the first new member of the genus *Henipavirus*, has provided a set of novel findings and new tools for the study of the henipaviruses and paramyxoviruses in general. CedPV is similar to both HeV and NiV, the canonical members of the genus *Henipavirus*, in the functional characteristics of their attachment and fusion glycoproteins, with both being required for membrane fusion as well being pH-independent (**Fig. 10**). However, CedPV is also unique in its extremely broad ephrin receptor usage tropism, where HeV and NiV are restricted to ephrin-B2 and ephrin-B3, but CedPV capable of employing ephrin-A1, ephrin-A2, ephrin-A5, ephrin-B1 and ephrin-B2 as functional fusion-triggering cellular receptors (**Fig. 13**). This is remarkable not only for the number of ephrin protein subtypes CedPV can utilize, but also for the fact that the A-type ephrins are GPI-linked, whereas the B-type ephrins are transmembrane anchored proteins. The broad protein receptor tropism exhibited by CedPV is the first example of a paramyxovirus capable of using both transmembrane-anchored and GPI-linked cellular proteins as receptors.

Additionally, the placement of CedPV within the genus *Henipavirus* is further supported by the observation that the F glycoprotein of CedPV is capable of functional cell-cell membrane fusion in heterotypic combination with the G glycoproteins of both HeV and NiV. Likewise, the G glycoprotein of CedPV is functional in combination with the F glycoprotein of HeV. But uniquely however, the heterotypic combination of CedPV G with NiV F is not functional for mediating cell-cell membrane fusion, and this establishes a novel system in which the F and G glycoproteins of three viruses within a single paramyxovirus genus exhibit imperfect bi-directional functionality. This experimental platform will afford a unique opportunity for a detailed examination of the determinants of the F and G interaction, receptor binding and selectivity, and fusion triggering activity by G, following receptor binding, making significant contributions to the study of the paramyxovirus fusion process.

Appendix

1. Codon optimized sequences of Cedar Virus

CedPV F (S-peptide tag sequence is underlined):

ATGAGCAACAAGCGGACCACTGTCCTGATTATTATCTCTTACACTCTGTTTTA
TCTGAACAACGCAGCCATCGTCGGGTTTGACTTCGACAAGCTGAACAAGATT
GGAGTGGTCCAGGGGAGGGTCCTGAACTACAAGATCAAAGGCGACCCCATG
ACCAAGGATCTGGTGCTGAAATTCATTCCTAACATCGTGAACATCACAGAGT
GCGTCCGAGAACCACTGAGCAGGTACAACGAGACTGTGAGGAGACTGCTGCT
GCCCATCCACAATATGCTGGGACTGTATCTGAACAATACCAACGCTAAGATG
ACAGGGCTGATGATCGCAGGCGTGATTATGGGCGGAATTGCCATCGGAATTG
CTACTGCCGCTCAGATCACCGCAGGGTTCGCCCTGTACGAGGCCAAAGAAAAA
CACTGAAAATATCCAGAAGCTGACCGACTCAATTATGAAAACACAGGACAGC
ATCGATAAGCTGACAGATTCAGTCGGGACTAGCATCCTGATTCTGAACAAGC
TGCAGACATACATCAACAATCAGCTGGTGCCAAATCTGGAGCTGCTGTCTTGT
CGGCAGAACAAAATCGAATTTGACCTGATGCTGACTAAGTATCTGGTGGATC
TGATGACCGTCATCGGACCCAACATCAACAACCCTGTGAACAAAGATATGAC
CATTCACTCTCTGAGTCTGCTGTTTCGACGGCAATTACGATATCATGATGTCCG
AGCTGGGATATACCCCCCAGGACTTTCTGGATCTGATCGAATCAAAGAGC
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CedPV G (S-peptide tag sequence is underlined):

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GCTGAGTTTTGGCTCACCTGGACGGCTGAGCAAAACCCTGGGACAGGTGCTG
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CedPV M ((S-peptide tag sequence is underlined)) :

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2. Cloned *P. alecto* AP3B1 homolog.

P. alecto AP3B1

GCAGCAATGTCCAGTAACAGCTTCGCTTACAATGAGCAGTCCGGGGGAGGAG
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